PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/11, 15/12	A1	(11) International Publication Number: WO 97/08307 (43) International Publication Date: 6 March 1997 (06.03.97)

(21) International Application Number: PCT/KR96/00145

(22) International Filing Date: 23 August 1996 (23.08.96)

(30) Priority Data:
1995/26391 24 August 1995 (24.08.95) KR

(71) Applicant (*for all designated States except US*): IL DONG PHARMACEUTICAL CO., LTD. [KR/KR]; 60, Yangjae-dong, Seocho-ku, Seoul 137-130 (KR).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): KIM, Sun, Young [KR/KR]; 3-306, Hanshin Seolae Apartment, 70-1, Panpo-dong, Seocho-ku, Seoul 137-040 (KR). KIM, Kee, Won [KR/KR]; 77-1402, Hyundai Apartment, 456, Apkujeong-dong, Kangnam-ku, Seoul 135-110 (KR). KIM, Tae, Han [KR/KR]; 206-1503, LG Apartment, 111, Jeongja-dong, Puntang-ku, Seongnam-shi, Kyonggi-do 463-010 (KR). HWANG, Jeong, Ho [KR/KR]; 203-1202, Hanshin Apartment, 124, Imae-dong, Puntang-ku, Seongnam-shi, Kyonggi-do 463-060 (KR). KIM, Seon, Hee [KR/KR]; 23-704, Hanshin 3rd Apartment, Panpo 2-dong, Seocho-ku, Seoul 137-042 (KR). LEE, Sun, Young [KR/KR]; 205, Samick Hights Villa, 38-3, Karak-dong, Songpa-ku, Seoul 138-160 (KR).

(74) Agent: KIM, Won, Ho; You Me Patent & Law Firm, 702, Teheran Boulevard, 825-33, Yoksam-dong, Kangnam-ku, Seoul 135-080 (KR).

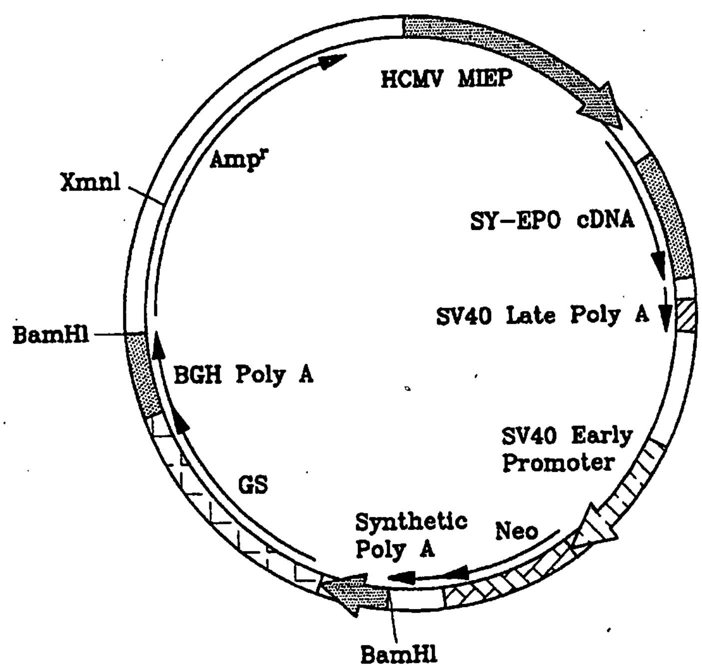
(81) Designated States: AU, CA, CN, JP, KR, MX, RU, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
With amended claims.

(54) Title: HETEROLOGOUS PROTEIN PRODUCTION SYSTEM USING AVIAN CELLS

(57) Abstract

Heterologous protein expression system including a heterologous gene DNA such as EPO genomic DNA, a vector receiving the DNA and an avian cell, such as duck embryo or quail fibrosarcoma cell line, expressing the gene in the vector can be used to efficiently produce heterologous proteins such as EPO.



ATTORNEY DOCKET NUMBER: 10165-022-999
SERIAL NUMBER: 10/612,665
REFERENCE: **B19**

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

HETEROLOGOUS PROTEIN PRODUCTION SYSTEM USING AVIAN CELLS

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to novel expression systems that can produce biomedically important heterologous proteins including human erythropoietin (hereafter "EPO"), and more specifically to the production of various heterologous proteins by transfecting DNA encoding the proteins, such as the genomic DNA encoding EPO into avian cells.

2. Related Arts

Many recombinant proteins used in medicine are relatively small and simple in their structure, and biologically functional proteins can be produced in prokaryote such as *E. coli*. However, some human proteins of medical interest, such as TPA (tissue plasminogen activator), Factor VIII, EPO, etc. are more complicated because biological function requires post-translational modification. For example, EPO is extensively glycosylated with the carbohydrate portion accounting for 40 % of the molecular mass. It has been shown that the carbohydrate portion of EPO is important for biological function. Accordingly, EPO produced in *E. coli*, yeast or insect is inactive or very weakly active *in vivo*, while EPO produced in COS or CHO cells was found to be fully active. Accordingly, those kinds of heterologous proteins have been produced only in mammalian cells.

In the meantime, the avian system has been used for the study of gene expression in higher eukaryote for a long time. One of the first viruses to be linked to tumors was the *Rous sarcoma* virus of chicken,

and this virus was instrumental in demonstrating that the retroviral oncogene can originate from a cellular gene, leading to the concept of the protooncogen. Studies of gene expression have also been done using the RSV LTR promoter, which has often be used for high level expression of heterologous genes in mammalian cells. In addition, avian embryo cells have been used extensively in studies of various animal viruses.

SUMMARY OF THE INVENTION

The present invention is a research for the high level expression of eukaryotic heterologous proteins. It is an object of the present invention to provide a novel heterologous gene expression system which can produce proteins of higher eukaryotic cells. It is another object to provide the method of efficiently producing higher eukaryotic proteins, such as EPO, etc., which has been known to be active only when they are produced in a mammalian cell. It is a further object of the invention to provide the method of producing, especially, EPO among the eukaryotic proteins described above.

To accomplish the objects of the present invention, the present invention provides a heterologous gene expression system comprising a DNA encoding a heterologous protein, a vector for receiving the DNA; and an avian cell for harboring the vector.

The present invention also provides a method of producing a heterologous protein comprising the steps of culturing the expression system of claim 1 in media to express the heterologous gene, and purifying the heterologous proteins from the cell and the media.

Preferably, the heterologous protein of the present invention is selected from the group consisting of those proteins that are known to be active only when expressed in mammalian cells (such as EPO, TPA,

Factor VIII, etc.) and preferably, the vector contains a promoter selected from the group consisting of SV early promoter, major immediate early promoter of human cytomegalovirus (hereafter "HCMV MIEP") and RSV LTR, and preferably, the avian cell is selected from the group consisting of duck embryo cell (hereafter "DE"), chicken embryo fibroblast (hereafter "CEF") and quail fibrosarcoma (hereafter "QT"), more preferably QT-VC which was isolated by the inventors. QT-VC was deposited to the International Depository Authority, Korea Research Institute of Bioscience and Biotechnology Korean Collection for Type Culture, and assigned a deposit number of KCTC 0277BP on August 22, 1996. The deposited QT-VC was transfected with the expression vector containing SY-EPO cDNA as described in Fig. 8.

More preferably, the DNA encoding the heterologous protein is genomic DNA or cDNA.

Further, the present invention provides an EPO production system comprising a DNA encoding EPO, a vector for receiving the DNA, and an avian cell for harboring the vector.

Moreover, the invention provides a method of producing EPO comprising the steps of inserting a DNA encoding EPO into a vector, transfecting the vector into an avian cell, and culturing the transfected avian cell in media.

Preferably, the avian cell of the EPO production system is DE or QT, and the DNA is a genomic DNA encoding EPO, more preferably, the DNA selected from the group consisting of SY, JM, SH and HE described in Fig. 5.

Preferably, the vector has a promoter selected from the group consisting of SV early promoter, HCMV MIEP and RSV LTR.

The present invention also provides an avian cell as a host for

expressing genes encoding mammalian proteins.

Further, the present invention provides an novel EPO genomic sequence selected from the group consisting of SY, JM, SH and HE described in Fig. 5, and also provides an novel EPO amino acid sequence selected from the group consisting of JM, SH and HE described in Fig. 6.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the expression of the bacterial CAT gene in avian cells. DE and CEF cells were transfected with pRc/CMV containing (+) or lacking (-) the CAT sequence. CAT activity was measured by determining the amount of acetylated chloramphenicol (AC) produced from ^{14}C-chloramphenicol. The values shown are from one representative of more than five independent assays. For this particular experiment, 10 μg of protein was reacted with ^{14}C-chloramphenicol for 20 min at 37 °C.

Fig. 2 shows the comparison of CAT gene expression between various cell types and between different promoters. The three promoter-CAT fusion constructs were transfected into DE, CEF, CHO-K1, and HeLa cells, and CAT activity was measured as described in Fig. 1. S, SV40 early promoter; C, HCMV MIEP; R, RSV LTR. The values shown are from one representative of three independent assays. For this particular experiment, 10 μg of protein was reacted with ^{14}C-chloramphenicol for 30 min at 37 °C .

Fig. 3 shows the efficiency of DNA transfection in various cells. pCMV-lacZ constructs was transfected into DE, CHO, Vero, HeLa, and 293T cells by calcium phosphate-DNA coprecipitation using the conditions used for the experiments shown in Fig. 2. Two days after transfection, cells were fixed and stained with X-gal. The number of

blue cells per 60 mm tissue culture plate was counted. The total number of cells between plates were comparable at 1-3 X 10^5. Transfection efficiency was calculated relative to DE cells.

Fig. 4 shows the schematic diagram for cloning of human EPO and construction of expression vectors. The five blocks represent the five coding regions of EPO. The first PCR was performed using primers 25 and 33. The amplified DNA fragment was cloned and subjected to a second PCR using primers 12 and 9. The wavy tale in primer 12 contains the nucleotide sequence from the first coding region. Therefore, the second PCR generates the entire coding sequence of EPO so that the first and the second coding regions are attached to form without intron between them. Primers 12 and 9 contain HindIII linkers at their 5' ends, enabling cloning of the EPO genomic sequence into various expression vectors.

Fig. 5 is various EPO genomic DNA sequences. SY, SH, HE and JM are the EPO genomic DNA sequences cloned by the present invention, and AM and GI are the EPO genomic sequences which has been already reported. Since the intron between the first coding region and the second coding region was deleted during the cloning, the deleted intron is not shown in Fig. 5.

Fig. 6 is various EPO amino acid sequences. SY, SH, HE and JM are the EPO amino acid sequences cloned by the present invention, and AM and GI are the EPO amino acid sequences which have been already reported. The abbreviation of the amino acids are as follows:

A: alanine R: arginine N: asparagine D: aspartic acid

C: cystein Q: glutamine E: glutamic acid H: histidine

I: isoleucine L: leucine K: lysine M: methionine

F: phenylalanine P: proline S: serine

T: threonine W: tryptophan Y: tyrosine V: valine

Fig. 7 shows the comparison of CAT gene expression between QT-VC and other mammalian cell lines. pCMV-CAT was transfected to QT-VC; CHO-K1, and Vero cells, and CAT activity was measured as described in Fig. 1. The transfection efficiency, as measured by X-gal staining following cotransfection with pCMV-lacZ, was reproducibly 3-5 % in all cases. For this particular experiment, 50 μg of protein were incubated with ^{14}C-chloramphenicol for one hour at 37 °C.

Fig. 8 is the typical structure of a plasmid used to express EPO in QT cells. The two types of BamHI cassettes which could express the gene for human glutamine synthetase (GS) was made. In these BamHI cassettes, the GS cDNA sequence was flanked by the poly A sequence from the bovine growth hormone gene and one of the two promoters, the partial MMTV LTR (from -220 to +15 from the RNA start site) or the 220 bp HSV tk promoter. The BamHI fragment expressing GS was inserted into the BamHI site of pCI-neo (Promega, Madison, WI, USA), resulting in a series of pIGA. The HindIII fragment of the SY-EPO cDNA sequence was cloned into the SmaI site of pIGA, generating the EPO expression vector, pIGA-EPO.

Fig. 9 shows the production of EPO by QT-N4D4. QT-N4D4 cells were grown to confluence in a 10 cm culture dish (day 0) in M-199 containing 10 % FBS and 1 mM MSX. On day 3, the EPO level was measured. The cells were then split into 1:3 and seeded onto 10 cm dishes. On day 6, the cells were again reached confluence, and the medium was replaced with 10 ml fresh medium containing 2 % (●) or 10 % (○) FBS. EPO levels were determined by ELISA (R & D system, Minnesota, USA)

Fig. 10 shows the comparison of EPO concentration in DE (●) and QT-N4D4 (○) measured by ELISA and by *in vitro* bioassay.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The inventors have explored the possibility of using avian cells as a host cell for heterologous gene expression. We have chosen to use three avian cells; two embryonic cells from chicken and duck, and a quail fibrosarcoma line. We chose to use the chicken and duck embryo cells for the following the reasons. First, these embryonic cells can easily be prepared from eggs, and they divide rapidly, undergoing many passages. Second, chicken and duck cells can be grown at large scale with relatively low costs. Third, some avian cells, such as those from chicken embryos have already been used for medical products. For example, influenza virus has been cultured in chicken eggs for the production of vaccines. Finally, the culture conditions, including media and temperature, required by avian embryo cells are virtually identical to those of mammalian cells, suggesting that the physiology of avian and mammalian cells is probably comparable. Further, the reason of choosing a QT cell line is that various transformed cell lines have been already developed, and it is easy to handle these cell lines to construct a permanent cell line expressing a heterologous protein, and the culture conditions and media is similar to those of mammalian cells.

I. Cells and Plasmids

1. Cells

The following Table 1 shows cells used in the experiment.

Table 1

Cells	Source
HeLa human cervical carcinoma cells	ATCC CCL2
Vero African green monkey kidney cells	ATCC CCL81
COS-7 African green monkey kidney cells transformed by wild-type T antigen of SV40	ATCC CRL1651
CHO-K1 Chinese hamster ovary cells	ATCC CCL61
NIH3T3 contacted-inhibited Swiss mouse embryo cells	ATCC CRL1651
Ad-5 transformed human embryonic kidney cells 293	ATCC CRL1651
SL-29 chicken embryo fibroblast cells	ATCC CRL1590
Duck embryo	ATCC CCL141 or prepared by the inventors
Quail fibrosarcoma line QT6	ATCC CRL1708
Quail fibrosarcoma line QT-VC	Isolated by the inventors KCTC 0277BP

All these cells except QT cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). QT cell lines were cultured in M199 medium instead of DEME. Duck embryo was either obtained from ATCC CCL 141 or prepared by trypsinization of 10- to 13- day old decapitated duck embryos. These avian cells were grown in minimum essential medium (Eagle) supplemented with non-essential amino acids and Earle's balanced salt solution containing 10 % FBS. These cells could be

maintained for approximately another 30 passages. Each medium used in this study was supplemented with 120 μg/ml penicillin G (Sigma P-3032; 1690 units per mg) and 200 μg/ml streptomycin (Sigma S-9137; 750 units per mg).

2. Plasmids

To evaluate the efficiency of heterologous protein production in avian cells, pRc/RSV-CAT and pRc/CMV-CAT were constructed by inserting a HindIII CAT cassette (Pharmacia, Piscataway, NJ) into the HindIII sites of pRc/RSV and pRc/CMV (Invitrogen, San Diego, California, USA), respectively. For pSVCAT, the plasmid p9l8 was used, which has been already described by the inventors. For EPO expression vectors, three vectors were used. pCMV-gEPO was constructed by cloning the HindIII fragments of the EPO genomic sequence into the HindIII site of pRc/CMV. pSV-gEPO was derived by replacing the CAT sequence of pSV918 with the genomic EPO sequence. pIGA-EPO has cDNA of EPO controlled by HCMV MIEP and the genes of NEO and glutamine synthetase (hereafter "GS"). To measure the transfection efficiency, the plasmid pCMV-lacZ was constructed by inserting bacterial lacZ fragment into the HindIII site of pRc/CMV.

II. DNA Transfection and Gene Expression Assays

The inventors tested whether avian embryo cells could be used for high levels of heterologous gene expression instead of mammalian cells. Although avian embryo cells have been used to culture viruses, there was no report that heterologous proteins of higher eukaryotic cells were expressed in these cells. To carry out the study, it is necessary to develop the method of efficient transfection to avian cells. That is, to express heterologous genes in avian cells, it is required to develop

the transfection technique of DNA to target cells. At present, we could not find any reports on DNA transfection of avian embryo cells. Accordingly, the inventors have developed the technique that CEF and DE cells can readily be transfected with DNA.

Among the techniques available, we have chosen a method using calcium phosphate coprecipitation, because this works well for various adherent cells and can also be used for establishing permanent lines. We have tested many different conditions and found that the following procedure was optimum.

When cultures were 50-70% confluent in a 100 mm culture dish, a total of 10 μg DNA in HBS buffer (140 mM NaCl, 5 mM KCl, 0.75 mM $Na_2HPO_4.2H_2O$, 6 mM dextrose, 25 mM HEPES) was incubated with the cells for 30 min at room temperature. 10 ml of regular media containing FBS was added and incubated for 20 hrs at 37 °C, except for CHO-K1 (8 hours). Cells were then treated with 10 ml of 100 μM chloroquine, and incubated for another 3 hours at 37 °C. After replacement with 10 ml of fresh media, the cells were grown for 1 to 2 days. Culture supernatants were collected and centrifuged at 1000 rpm for 10 min to remove cells and debris. To measure transfection efficiency, cells were transfected with pCMV-lacZ, rinsed once with PBS 3 days after transfection, fixed with 0.5 % glutaraldehyde (in PBS) for 10 min, and washed twice for 2-10 min each with 4 ml PBS containing 1 mM $MgCl_2$. For X-gal staining, the staining solution [PBS containing 4 mM $K_3Fe(CN)_6$, 4 mM $K_4Fe(CN)_6.3H_2O$, 2 mM $MgCl_2$, and 400 μg per ml X-gal (in dimethylformamide)] was added to fixed cells, and incubated at 37 °C for 4 hours overnight. When the reaction was completed, cells were washed once with PBS. Stained cells were kept in PBS.

CAT assay was carried out as follows:

Two to three days after transfection, cells were harvested, washed once with PBS, and resuspended in 0.25 M Tris-HCl (pH 7.5). Total proteins were prepared by 4 cycles of freeze/thawing followed by heating at 65 °C for 7 min. Equivalent amounts of protein were assayed for CAT activity at 37 °C for 30 min. The amount of protein and the reaction time varied, depending on the experiments. For example, the CAT activity of cell extracts prepared from DE cells was so high that only 10 μg protein and 20 to 30 min reaction time had to be used, and under this condition, levels of CAT activity in other mammalian cells were very low or undetectable. When CAT activity became detectable in other cells, virtually all ^{14}C-chloramphenicol was converted. The percent conversion of ^{14}C-chloramphenicol to its acetylated forms was determined by cutting out regions containing unreacted and acetylated forms and quantifying the amount of radioactivity in each by liquid scintillation counting.

III. Gene Expression in DE and CEF

Gene expression efficiency of DE and CEF was measured using CAT gene. We initially chose to use a promoter from the major immediate-early region of HCMV, because this has been shown to drive a high level gene expression in many different cell types. In the plasmid pCMV-CAT, the bacterial CAT gene is placed under the control of the HCMV MIEP. As a negative control, the plasmid Rc/CMV containing the promoter but no CAT sequence was used. These plasmids were transfected into DE and CEF cells and CAT activity was measured to estimate the efficiency of transfection and gene expression. One representative result from several independent transfections is shown in Fig. 1. Transfection of a control plasmid resulted in undetectable levels of CAT activity in both cells. However,

transfection with pCMV-CAT resulted in readily detectable levels of CAT activity in both cells. In more than five independent transfection assays, the level of CAT activity was always higher in DE cells than in CEF cells. The magnitude of difference in the level of CAT activity between the two cells ranged from 10- to 50-fold, depending on the experiment. This result indicated that avian cells were readily transfected with DNA and the heterologous genes could be efficiently expressed.

IV. Comparison of Levels of Gene Expression between Avian and Mammalian Cells, and between Different Promoters

We have compared the levels of gene expression between avian and mammalian cells, using three different promoters;

(1) the SV40 early promoter, which is used during the early transcriptional phase of SV40 infection;

(2) the HCMV MIEP, which drives the expression of IEI and IE2 regulatory proteins, immediately after HCMV infection;

(3) the RSV LTR from an avian retrovirus.

These promoters are known to be powerful in mammalian cells, and have often been used for high level heterologous gene expression.

These promoter-CAT fusion constructs were transfected into four different cell lines, DE, CEF, CHO-K1, and HeLa, and CAT activity measured to compare the efficiency of gene expression between promoters and between cell types. To make this comparison semi-quantitative, all transfections and CAT assays were performed at the same time and using identical conditions. One representative result of such experiments is shown in Fig. 2. Here, 10 μg of cell extracts were incubated for 30 min in the CAT reaction. Under these

particular conditions, the levels of CAT expression driven from the three promoters were very low in CHO and HeLa cells (Fig. 2). Only after larger amounts of proteins were used for extended reaction time, was CAT activity detected. In contrast, CAT activity was readily detectable in the avian cells (Fig. 2), except for the SV40 promoter in CEF cells. It indicated that the expression in avian cells are more effective than that in mammalian cells.

The most dramatic finding was that the HCMV MIEP was extremely powerful in DE cells. In Fig. 2, the conditions used for the CAT reaction were chosen to generate the reasonable levels of CAT activity in other samples. When the CAT reaction was performed under limiting conditions for the protein sample prepared from DE cells transfected with pCMV-CAT (i.e., when the CAT conversion was below 50 %), the levels of CAT activity of all the other samples were virtually undetectable. Therefore, the magnitude of difference in CAT activity between the protein sample from DE cells transfected with HCMV-CAT and those from the other transfections is at least two orders of magnitude. These results suggested that heterologous genes could be expressed very efficiently under the control of the HCMV MIEP in DE cells.

It is possible that the high levels of CAT expression seen in DE cells could be due to efficient transfection of the cell population, rather than an ability of these cells to support strong gene expression. To distinguish these possibilities, we transfected pCMV-lacZ into DE and various animal cells. After transfection, cells were stained with X-gal, and the number of blue cells were counted to estimate the transfection efficiency. As shown in Fig. 3, the number of stained cell was always comparable between DE and other animal cells, suggesting that the high levels of CAT expression in DE cells were due to high levels of

expression in individual cells.

V. Cloning of human erythropoietin

To test whether DE cells could indeed be used for the expression of medically important human proteins, we have isolated the genomic DNA encoding the human EPO gene. We chose to use EPO as a model because it is a secreted protein, so we could test whether DE cells properly process secreted proteins. We also used a genomic clone of EPO instead of the cDNA, to assess whether human genes are properly spliced to produce functional mRNAs in DE cells.

DNAs for cloning of EPO were prepared with blood cells collected from four people. Human peripheral blood lymphocytes were isolated by Ficoll-Hypaque gradient centrifugation of heparin-treated blood cells. Total DNA was prepared and used for polymerase chain reaction using specific oligonucleotide primers (Fig. 4). The region around the start codon was highly GC rich, so the EPO sequence was cloned by two steps of PCR using two different pairs of primers.

To obtain the genomic DNA for EPO, total DNA was prepared by lysing human peripheral blood lymphocytes using TES (10 mM Tris-HCl pH 7.8; 1 mM EDTA; 0.7 % SDS) followed by the treatment with 400 μg/ml proteinase K at 50 °C for 1 hour, phenol:chloroform extraction, and ethanol precipitation. The polymerase chain reaction (PCR) was performed using 0.1 μg of total genomic DNA and oligonucleotide primers specific to the EPO gene.

Primer #25 (sense, 5' to 3'): GAAGCTGATAAGCTGATAACC

Primer #33 (antisense, 5' to 3'): TGTGACATCCTTAGATCTCA

The samples were amplified through 30 cycles that included the

following parameters; denaturation at 92 °C for 1 min, primer annealing at 55 °C for 1min, and primer extension at 72 °C for 1 min. The DNA fragment amplified from this reaction did not contain the first 13 nucleotides in the N-terminal region, so a second PCR was performed using the following primers (Underlined, HindIII; Outlined, start codon and stop codons, respectively). The relative position of these primers are as shown in Fig. 4. Taq DNA polymerase (POSCO Chem, Korea) and pfr polymerase (STRATGENE, California, USA) were used to amplify DNA.

Primer #12 (sense, 5' to 3'):

CAAGCTTCGGAGATGGGGTGCACGAATGTCCTGCCTGGCTGTGGC

Primer #9 (antisense, 5' to 3'):

CAAGCTTTCATCTGTCCCCTGTCCTGC

The amplified DNA from the second PCR was cloned into the pCRII (Invitrogen), from which the HindIII fragment containing the genomic sequence of EPO was inserted into various expression vectors as described above. In this experiment, the amplified DNA was placed under the control of the HCMV MIEP or SV40 early promoter, generating pCMV-gEPO and pSV-gEPO respectively. SY-EPO whose amino acid sequence is identical to that of the already known EPO is used for the expression experiments in the sections VII and VIII (See the section VI).

VI. Analysis of Nucleotide Sequences of Cloned EPO Genomes

Genomic structure of EPO cloned by the above method is different from the natural EPO genome *in vivo*. That is, wild type EPO genomic DNA has five coding regions and four introns between them. However, in the DNA cloned by the above method, the first coding region was fused

to the second coding region to form one coding region so that it has four coding regions and three introns (Fig. 4).

The results from the analysis of EPO gene sequences isolated from four people suggested that nucleotide sequences of EPO cloned from these region are significantly different from those of the prior two EPOs (AM-EPO and GI-EPO) (Fig. 5) at the sites of intron. Such a difference was not due to the error which occurred during DNA amplification in the process of cloning. We repeated cloning and sequencing using DNAs prepared from same individuals (but at different times) and obtained the same nucleotide sequence. As another control, we amplified the already cloned EPO under the similar conditions, and determined the nucleotide sequence. Again, we obtained the same nucleotide sequence.

Amino acid sequences of four EPO genes, together with AM and GI, are shown in Fig. 6. Amino acid sequences from AM, GI and SY are identical. However, amino acid sequences from three people (JM, SH, HE) different by two or three different amino acids from GI- and AM-EPO, suggesting that there is a polymorphisms among people. When compared with AM- or GI-EPO, HE-EPO had three different amino acids at C-terminal, SH-EPO three different amino acid over the whole polypeptide, and JM-EPO two different amino acids, one at C-terminal and the other in the middle of polypeptide (See Fig. 6). For example, while AM-EPO and GI-EPO had serine, alanine, and valine at positions 36, 100 and 170 respectively, SH-EPO had arginine, serine, and tyrosine. Further, while AM-EPO and GI-EPO had valine, lysine, and aliginine at positions 170, 177, and 191, HE-EPO had tyrosine, glutamine, and glycine. In JM-EPO, lysine and tyrosine were present at positions 54 and 170, while they were threonine and valine. These results suggested that the EPO gene has a polymorphism in amino

acids sequence as well as DNA sequence.

VII. Expression of EPO in DE Cells

In this experiment, we compared levels of EPO expression between DE cells and other cell lines.

EPO expression vectors were transfected into various cells including DE, CEF, CHO, HeLa, VERO, and 293T. We have included VERO cells because they are often used for heterologous gene expression, and 293T cells which drive very high levels of gene expression, presumably due to both the high frequency of DNA transfection and the presence of potent viral transactivators such as EIA, EIB, and large T antigen. Two to three days after transfection, levels of EPO in the culture supernatants were measured by the enzyme linked immunoadsorbent assay, and transfection efficiencies were determined by staining cells adhered on the culture with X-gal. Transfection efficiency was carried out by transfection of a lacZ expression vector together with an EPO expression vector as described in the section II. One representative result of this analysis is summarized in Table 2.

Table 2

Cell	HCMV MIEP	SV40 early promoter	HCMV/SV40
293	314	17.5	18
CHO	139.4	10.4	13.5
VERO	250	10.7	23.5
NIH3T3	89	79.4	1.1
DE	4335	13.8	314.8

When the SV40 early promoter was used, there was little

difference in the levels of EPO between cell types. However, when the HCMV MIEP was used, DE cells produced much higher levels of EPO than any other cell lines tested. The HCMV MIEP was much more active than the SV40 early promoter in almost all the cells tested. This difference was especially pronounced in DE cells, where the former produced 315 times more EPO than the latter. Among the various cell types, DE cells always produced the highest level of EPO. CHO cells are the source of cell lines producing EPO that is currently used for human application. In this transient system, however, the level of EPO in CHO cells was at least 30-fold lower than in DE cells. Difference between DE and 293T cells was also considerable. Transfection efficiency of 293T was higher by about 30-folder than any other cells including DE cells. Moreover, 293T cells produce potent viral transcription transactivators. Nevertheless, DE produced 10-folder more EPO than 293T, suggesting that DE could drive high levels of the gene expression.

In conclusion, human EPO could efficiently be produced and secreted in DE cells and that the HCMV MIEP is the promoter of choice for driving high level heterologous gene expression in DE cells.

In summary, we found that DE cells could produce very high levels of bacterial and human proteins. All three promoters tested drove higher levels of gene expression in DE cells than any other cell lines used in this study. In particular, the HCMV MIEP was extremely powerful in DE cells. The high level of heterologous gene expression observed was not due to a higher number of transfected cells. It appears that DE cells properly process splicing and secretion because transfection of DE cells with an expression vector containing the EPO genomic DNA sequence produced a large quantity of EPO in the culture supernatant.

For DE cells to be used for industrial purpose, one would need to develop large-scale culture techniques for these cells. There are two possible ways. First, it may be possible to use primary cells themselves as the producer line. A large number of DE cells can easily be prepared from 10- to 13 day-old duck embryos. From one embryo, we can readily obtain 10^9 to 10^{10} cells that can undergo at least 15 passages. Therefore, it is possible to transfect DE cells at the earliest possible stage with an expression vector followed by selection of transfected cells, which might require 4-7 passages. Even if less than 5% of the cells were transfected, a large number of transfected cells would be available, suggesting that large-scale culture of primary duck embryo cells is not impossible with primary cells. Second, it will be possible to transform duck embryo cells at an early stage, using one of the large number of well-characterized oncogenes that are available. With transformed DE cells, a producer line could be constructed, and better quality control of protein production be established. It remains to be seen whether transformed DE cells will still maintain the capability for high level gene expression. Although a number of biological questions remain to be answered, the potential of these cells for the production of various proteins warrants further investigation.

VIII. Heterologous Gene Expression in the Transformed Avian Cell Line

The above experiments demonstrated the great potential of DE cells as producers of heterologous proteins such as EPO. However, DE cells used in the above experiments are primary cells and stop dividing after 30-40 passages *in vitro*. Therefore, unless DE cells are transformed or special techniques are developed as described above, it is difficult to use these embryonic cells for industrial production of heterologous proteins.

In the following study, we tested whether the transformed avian cell line, namely the quail fibrosarcoma line, could be used to produce EPO. The quail fibrosarcoma line used in this study, QT-VC, was subcloned from QT6 (ATCC CRL1708). This line was derived from methylcholanthrene-induced fibrosarcoma of Japanese quail. QT-VC is different from its parental line in at least two aspects. First, QT-VC grows faster than the parental line in M199 medium containing 10% FBS used in this study. The former divided every 12-24 hours, while the doubling time of the latter was 24-36 hours. Second, the QT-VC cell looks more roundish than QT6 which generally grows in a longish form. Like its parental line, QT-VC did not grow well when it was seeded at a low density. Therefore, cells had to be split to 1/3 to 1/2 after reaching confluence for continuous culture.

1. Analysis of Gene Expression in QT-VC Cells

We compared the levels of gene expression between QT-VC and mammalian cells using pCMV-CAT. We chose to use the HCMV MIEP as this promoter was shown to drive high levels of gene expression in various cell types including avian cells (See the section IV). pCMV-CAT was transfected into 3 cell lines, QT-VC, CHO-K1 and Vero. To make this comparison semi-quantitative, all transfections and CAT assays were performed at the same time and using identical conditions. Transfection efficiency was also measured by cotransfecting pCM-lacZ followed by X-gal staining. The efficiency was approximately 3 % in all cases. Under these conditions, the levels of CAT expression in QT-VC cells were always 2-3 times higher than mammalian cell lines used in this study (Fig. 7). Although the level of gene expression in QT-VC cells appears to be lower than DE cells, the quail fibrosarcoma line is at least as good as mammalian cell lines, suggesting that it could be used as a producer for heterologous

proteins.

2. Construction of EPO Expression Vectors for QT-VC Cells

To test whether high levels of heterologous proteins could be expressed in QT cells, we have constructed other EPO expression vectors. The basic strategy for the construction of an expression vector was as follows:

First, we chose to use the HCMV MIEP to drive expression of the heterologous gene as it had already been shown to be one of the strongest promoters in avian cells as well as mammalian cells.

Second, the human glutamine synthetase (GS) gene was used for amplification of the target gene. Generally, the gene of interest is amplified to augment the yield of protein by using certain selectable markers in the presence of specific chemicals. One of the best examples is the dihydrofolate reductase (DHFR) gene. It has been shown that the copy number of the heterologous gene and the level of respective protein increase as the concentration of methotrexate (MTX) in the medium is slowly increased. However, this system requires the host cell defective in the gene DHFR, so cannot be directly applied to QT cells for which such a mutant line is not yet available. For this reason, we chose to use the GS gene. In this case, the host cell line need not to be deficient for GS, because only multiple copies of the GS gene can confer resistance to methionine sulfoximine (MSX).

The overall structure of EPO expression vectors constructed for the use in QT cells is shown in Fig. 8. In this structure, the cDNA sequence for EPO is under the control of the HCMV MIEP, the bacterial Neo gene is used as the first selectable marker, and the human GS gene is also present as the second selectable marker in the same plasmid. The backbone of expression vectors used in this particular

experiment was pCI-neo (Promega, USA) which uses the HCMV MIEP and the intron from the β-globin genome. We have made a couple of different constructs in which the GS gene is driven by the partial MMTV LTR (from -220 to +15) or the 220 bp HSV tk promoters. In either case, the magnitude of gene amplification appears to be comparable (Data not shown). Detailed procedure and supplementary data regarding the construction of expression vectors is available upon request.

3. Construction of QT-VC Cells Stably Expressing EPO

To construct QT-VC cell lines constitutively expressing EPO, the cells were transfected with an EPO expression vector by a calcium phosphate coprecipitation method as described in the section II. Three days after transfection, EPO production was confirmed by EILSA and transfected cells were treated with G418 (0.8 mg/ml) and MSX (25 μM). When G418-resistant cells were grown to confluence, cells were diluted for sublconing. Because QT-VC cells do not grow efficiently at a low cell density, cells were seeded on 10-cm culture dishes at various numbers (10^2, 10^3, 10^4, 10^5 per dish). Then the colonies that grew distant from other colonies were isolated by plastic O rings and expanded onto a 96-well plate. When cells reached 70 % confluence, the EPO level was measured. Subclones that produced more than 200 U/ml were serially expanded from the 12-well to 6-well to 60 mm culture plates. When cells reached confluence on a 60 mm dish, cells were split on 6-well plates and then treated with various concentrations of MSX (100 μM, 250 μM, 1 mM). Using this procedure, several subclones that produced large amounts of EPO and also grew fast were selected.

One of the subclones obtained through this procedure is QT-

N4D4. As shown in Fig. 9, this subclone produced 1200 U/ml when grown for 3 days after confluence. When the cells were split to 1:3, seeded on 10 cm dishes, and allowed to grow for another 3 days, N4D4 still produced 1000 U/ml. The medium was then replaced with a fresh media containing 2 % FBS and the cells still produced 400 U/ml EPO. These results indicated that QT cells could produce a large quantity of EPO.

In conclusion, the above experiment demonstrated the great potential of QT cells as a producer for heterologous protein.

IX. Biological Activity of EPO Produced in Avian Cells

EPO is heavily glycosylated and such glycosylation is required for its biological activity. For example, EPO produced in *E. coli* or yeast is inactive or very weakly active *in vivo*. To test whether EPO expressed in DE or QT cells was biologically active, we carried out an *in vitro* bioassay using spleen cells isolated from mice treated with phenylhydrazine.

EPO assay: Absolute levels of EPO production after transfection of various cells were determined by enzyme linked immunoadsorbent assay which is currently used to, measure EPO levels in the human serum (R & D Systems Inc., Minnesota, USA). To measure the biological activity of EPO, *in vitro* bioassay was carried out by the method of Krystal as modified by Goldberg et al Spleen cells were taken from C57BL X C3H Fl hybrid mice (Seoul National University Laboratory Animal Center) on day 3 after the second of two daily injections of phenylhydrazine (60 mg/Kg of body weight per day) and spleen cell suspensions were prepared with Lymphoprep™ (NYCOMED PHARMA AS, Oslo, Norway). The spleen cells (final concentration 4×10^6 cells per ml) were then incubated in 24 well tissue

culture plates with various standard doses of EPO (CILAG AG International, Switzerland; specific activity 2000 U/ml) or unknown samples for 22 hr and then pulsed with 4 μCi/well tritiated thymidine (Amersham Co.) for 2-3 hr. The cells were harvested, washed with PBS several times and lysed by 0.3 N NaOH and 0.1% SDS. Radioactivity in LSC cocktail solutions were calculated by a Pharmacia Wallac 1410 scintillation counter.

Culture supernatants from QT-N4D4 cells or DE cells transfected with EPO expression vectors were taken to measure levels of EPO by both ELISA and the bioassay. The ELISA measures absolute concentration, and is currently used for determining EPO concentration in human serum. On the other hand, the bioassay determines biological activity using a control EPO that has been produced from mammalian cells and is currently being used in humans. Fig. 10 compares the difference in levels of EPO determined by these two methods. The ratio between the values (mU) was I ± 0.15, and the specific activity of EPO produced from DE cells was estimated to 105 U/μg. Therefore, the levels of EPO measured by ELISA were very comparable to those obtained by the bioassay. This result suggested that EPO produced from these avian cells had a similar biological activity to commercially available EPO.

What is claimed is:

1. A heterologous gene expression system comprising:

a DNA encoding a heterologous protein;

a vector for receiving the DNA; and

an avian cell for harboring the vector.

2. The expression system of claim 1, wherein the heterologous protein is selected from the group consisting of TPA, Factor VIII and EPO.

3. The expression system of claim 1, wherein the vector contains a promoter selected from the group consisting of SV early promoter, HCMV MIEP and RSV LTR.

4. The expression system of claim 1, wherein the avian cell is selected from the group consisting of DE, CEF and QT.

5. The expression system of claim 4, wherein the QT is QT-VC.

6. The expression system of claim 1, wherein the DNA encoding the heterologous protein is DNA or cDNA.

7. An avian cell as a host for expressing a gene encoding a mammalian heterologous protein.

8. A method of producing a heterologous protein comprising the steps of;

culturing the avian cell containing the expression system of claim 1 to express the gene of the heterologous protein in media; and

purifying the heterologous protein from the cell and the media.

9. The method of claim 8, wherein the heterologous protein is selected from the group consisting of TPA, Factor VIII and EPO.

10. The method of claim 8, wherein the vector contains a promoter selected from the group consisting of SV early promoter, HCMV MIEP and RSV LTR.

11. The method of claim 8, wherein the avian cell is selected from the group consisting of DE, CEF and QT.

12. The method of claim 11, wherein the QT is QT-VC.

13. The method of claim 8, wherein the DNA encoding the heterologous protein is DNA or cDNA.

14. An EPO production system comprising:

a DNA encoding EPO;

a vector for receiving the DNA; and

an avian cell for harboing the vector.

15. The EPO production system of claim 14, wherein the avian cell is DE or QT.

16. The EPO production system of claim 15, wherein the QT is QT-VC.

17. The EPO production system of claim 14, wherein the DNA is a genomic DNA encoding EPO.

18. The EPO production system of claim 14, wherein the DNA encoding EPO is selected from the group consisting of SY, JM, SH and HE described in Fig. 5.

19. The production system of claim 14, wherein the vector contains a promoter selected from the group consisting of SV early promoter, HCMV MIEP and RSV LTR.

20. A method of producing EPO comprising the steps of:

inserting a DNA encoding an EPO into a vector;

transfecting the vector into an avian cell; and

culturing the transfected avian cell in media.

21. The method of claim 20, wherein the avian cell is DE or QT.

22. Them method of claim 21, wherein the QT is QT-VC.

23. The method of claim 20, wherein the DNA encoding EPO is a genomic DNA.

24. The method of claim 20, wherein the DNA encoding the EPO is selected from the group consisting of SY, JM, SH and HE described in Fig. 5.

25. The method of claim 20, wherein the vector contains a promoter selected from the group consisting of SV40 early promoter, RSV LTR and HCMV MIEP.

26. An EPO genomic sequence selected from the group consisting of SY, JM, SH and HE described in Fig. 5.

27. An EPO amino acid sequence selected from the group consisting of JM, SH and HE described in Fig. 6.

FIG.1

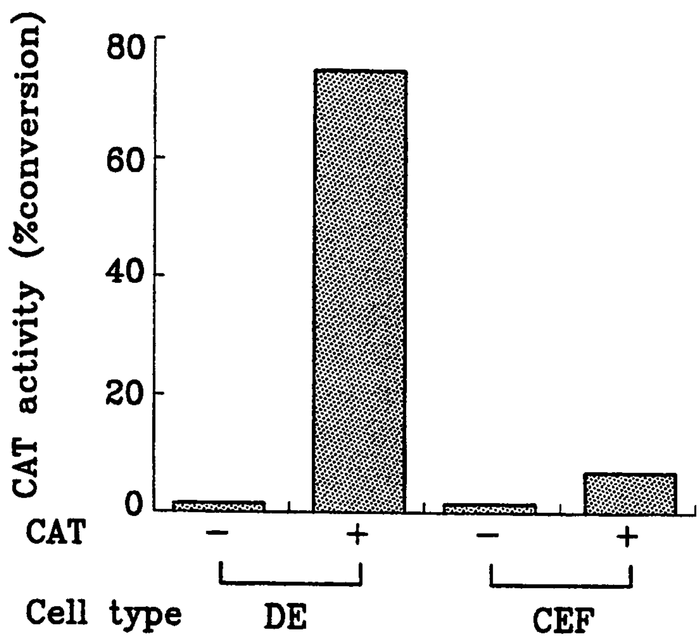


FIG.2

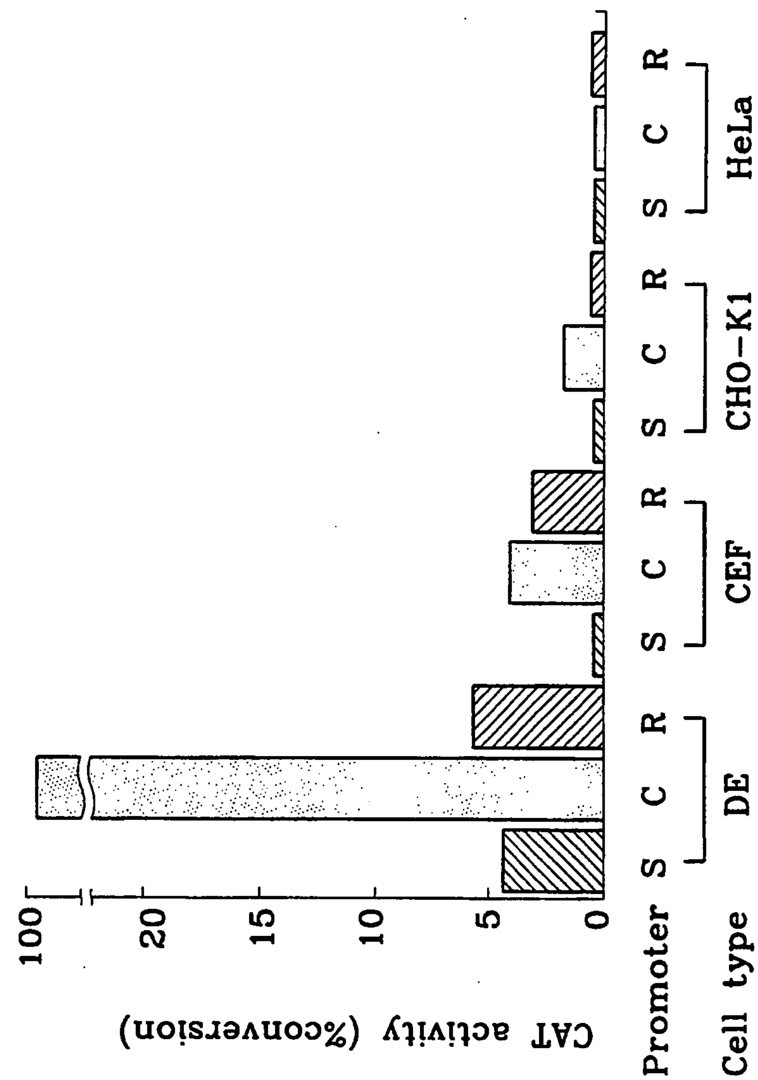

CAT activity (%conversion)
100
20
15
10
5
0
Promoter
S
C
R
S
C
R
S
C
R
S
C
R
Cell type
DE
CEF
CHO-K1
HeLa

FIG.3

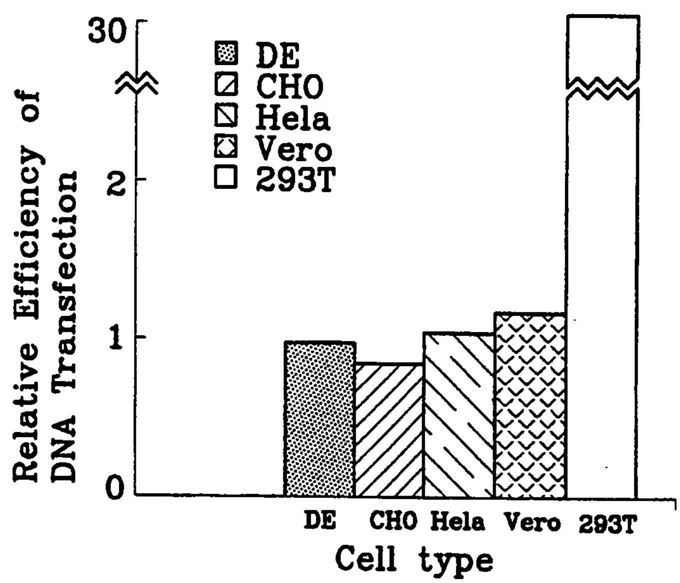

30
2
1
0
DE
CHO
Hela
Vero
293T
Relative Efficiency of
DNA Transfection
DE
CHO
Hela
Vero
293T
Cell type

FIG.4

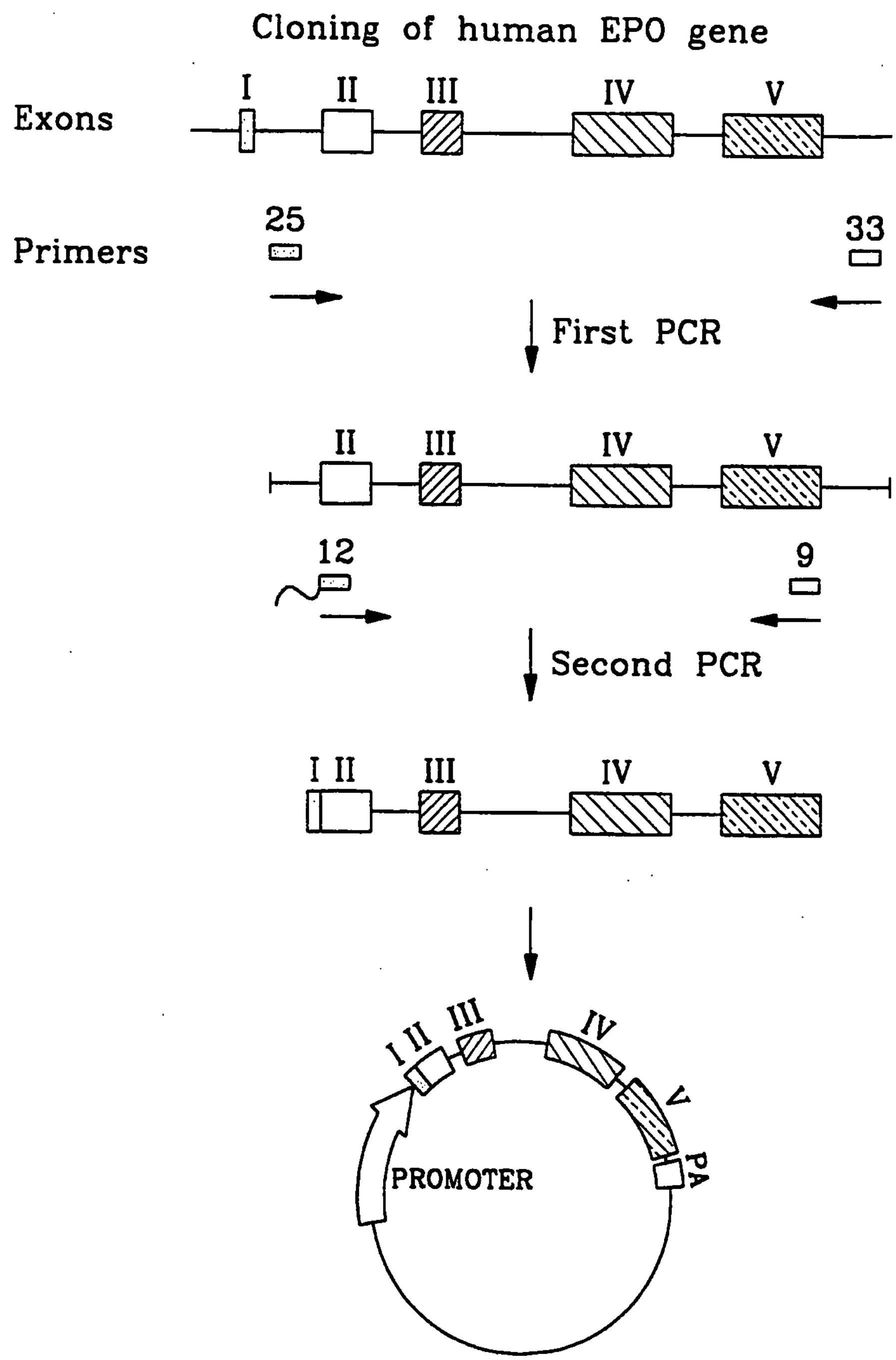

Cloning of human EPO gene
Exons
I
II
III
IV
V
Primers
25
33
First PCR
II
III
IV
V
12
9
Second PCR
I II
III
IV
V
I II
III
IV
V
PA
PROMOTER

FIG.5A

```
AM    ATGGGGGTGCACGAATGTCCTGCCTGGCTGTGGCTTCTCCTGTCCCTGCT    50
GI    ATGGGGGTGCACGAATGTCCTGCCTGGCTGTGGCTTCTCCTGTCCCTGCT    50
SY    ATGGGGGTGCACGAATGTCCTGCCTGGCTGTGGCTTCTCCTGTCCCTGCT    50
JM    ATGGGGGTGCACGAATGTCCTGCCTGGCTGTGGCTTCTCCTGTCCCTGCT    50
SH    ATGGGGGTGCACGAATGTCCTGCCTGGCTGTGGCTTCTCCTGTCCCTGCT    50
HE    ATGGGGGTGCACGAATGTCCTGCCTGGCTGTGGCTTCTCCTGTCCCTGCT    50
      **************************************************

AM    GTCGCTCCCTCTGGGCCTCCCAGTCCTGGGCGCCCCACCACGCCTCATCT    100
GI    GTCGCTCCCTCTGGGCCTCCCAGTCCTGGGCGCCCCACCACGCCTCATCT    100
SY    GTCGCTCCCTCTGGGCCTCCCAGTCCTGGGCGCCCCACCACGCCTCATCT    100
JM    GTCGCTCCCTCTGGGCCTCCCAGTCCTGGGCGCCCCACCACGCCTCATCT    100
SH    GTCGCTCCCTCTGGGCCTCCCAGTCCTGGGCGCCCCACCACGCCTCATCT    100
HE    GTCGCTCCCTCTGGGCCTCCCAGTCCTGGGCGCCCCACCACGCCTCATCT    100
      **************************************************

AM    GTGACAGCCGAGTCCTGGAGAGGTACCTCTTGGAGGCCAAGGAGGCCGAG    150
GI    GTGACAGCCGAGTCCTGGAGAGGTACCTCTTGGAGGCCAAGGAGGCCGAG    150
SY    GTGACAGCCGAGTCCTGGAGAGGTACCTCTTGGAGGCCAAGGAGGCCGAG    150
JM    GTGACAGCCGAGTCCTGGAGAGGTACCTCTTGGAGGCCAAGGAGGCCGAG    150
SH    GTGACAGACGAGTCCTGGAGAGGTACCTCTTGGAGGCCAAGGAGGCCGAG    150
HE    GTGACAGCCGAGTCCTGGAGAGGTACCTCTTGGAGGCCAAGGAGGCCGAG    150
      ******* ******************************************

AM    AATATCACGGTGAGACCCCTTCCCCAGCACATTCCACAGAACTCACGCTC    200
GI    AATATCACGGTGAGACCCCTTCCCCAGCACATTCCACAGAACTCACGCTC    200
SY    AATATCACGGTGAGACCCCTTCCCCAGCACATTCCACAGAACTCACGCTC    200
JM    AATATCACGGTGAGACCCCTTCCCCAGCACATTCCACAGAACTCACGCTC    200
SH    AATATCACGGTGAGACCCCTTCCCCAGCACATTCCACAGAACTCACGCTC    200
HE    AATATCACGGTGAGACCCCTTCCCCAGCACATTCCACAGAACTCACGCTC    200
      **************************************************
```

FIG.5B

```
AM    AGGGCTTCAGGG-AACTCCTCCCAG-ATCCAGGAACCTGGCACTTGGTTT   248
GI    AGGGCTTCAGGG-AACTCCTCCCAG-ATCCAGGAACCTGGCACTTGGTTT   248
SY    AGGGCTTCAGGG-AACTCCTCCCAG-ATCCAGGAACCTGGCACTTGGTTT   248
JM    AGGGCTTCAGGG-AACTCCTCCCAG-ATCCAGGAACCTGGCACTTGGTTT   248
SH    AGGGCTTCAGGG-AACTCCTCCCAG-ATCCAGGAACCTGGCACTTGGTTT   248
HE    AGGGCTTCAGGGGAACTCCTCCCAGGATCCAGGAACCTGGCACTTGGTTT   250
      ************ ************ ************************

AM    GGGGTGGAGTTGGGAAGCTAGACACTGCCCCCCTACATAAGAATAAGTCT   298
GI    GGGGTGGAGTTGGGAAGCTAGACACTGCCCCCCTACATAAGAATAAGTCT   298
SY    GGGGTGGAGTTGGGAAGCTAGACACTGCCCCCCTACATAAGAATAAGTCT   298
JM    GGGGTGGAGTTGGGAAGCTAGACACTGCCCCCCTACATAAGAATAAGTCT   298
SH    GGGGTGGAGTTGGGAAGCTAGACACTGCCCCCCTACATAAGAATAAGTCT   298
HE    GGGGTGGAGTTGGGAAGCTAGACACTGCCCCCCTACATAAGAATAAGTCT   300
      **************************************************

AM    GGTGGCCCCAAACCATACCTGGAAACTAGGCAAGGAGCAAAGCCAGCAGA   348
GI    GGTGGCCCCAAACCATACCTGGAAACTAGGCAAGGAGCAAAGCCAGCAGA   348
SY    GGTGGCCCCAAACCATACCTGGAAACTAGGCAAGGAGCAAAGCCAGCAGA   348
JM    GGTGGCCCCAAACCATACCTGGAAACTAGGCAAGGAGCAAAGCCAGCAGA   348
SH    GGTGGCCCCAAACCATACCTGGAAACTAGGCAAGGAGCAAAGCCAGCAGA   348
HE    GGTGGCCCCAAACCATACCTGGAAACTAGGCAAGGAGCAAAGCCAGCAGA   350
      **************************************************

AM    TCCTACGGCCTGTGGGCCAGGGCCAG-AGCCTTCAGGGACCCTTGACTCC   397
GI    TCCTAC-GCCTGTGG-CCAGGGCCAG-AGCCTTCAGGGACCCTTGACTCC   395
SY    TCCTACGGCCTGTGGGCCAGGGCCAA-AACCTTCAGGGACCCTTGACTCC   397
JM    TCCTACGGCCTGTGGGCCAGGGCCAGGAGCCTTCAGGGACCCTTGACTCC   398
SH    TCCTACGGCCTGTGGGCCAGGGGCA-GAGCCTTCAGGGACCCTTGACTCC   397
HE    TCCTACGGCCTGTGGGCCAGGGCCA-GAGCCTTCAGGGACCCTTGACTCC   399
      ****** ******** ****** **  * *********************
```

FIG.5C

```
AM   CCGGGCTGTGTGCATTTCAGACGGGCTGTGCTGAACACTGCAGCTTGAAT   447
GI   CCGGGCTGTGTGCATTTCAGACGGGCTGTGCTGAACACTGCAGCTTGAAT   445
SY   CCGGGCTGTTTGCATTTCAGACGGGCTGTGCTGAACACTGCAGCTTGAAT   447
JM   CCGGGCTGTGTGCATTTCAGAAGGGCTGTGCTGAACACTGCAGCTTGAAT   448
SH   CCGGGCTGTGTGCATTTCAGACGGGCTGTGCTGAACACTGCAGCTTGAAT   447
HE   CCGGGCTGTGTGCATTCCAGACGGGCTGTGCTGAACACTGCAGCTTGAAT   449
     ********* ****** **** ****************************

AM   GAGAATATCACTGTCCCAGACACCAAAGTTAATTTCTATGCCTGGAAGAG   497
GI   GAGAATATCACTGTCCCAGACACCAAAGTTAATTTCTATGCCTGGAAGAG   495
SY   GAAAATATCACTGTCCCAGACACCAAAGTTAATTTCTATGCCTGGAAGAG   497
JM   GAGAATATCACTGTCCCAGACACCAAAGTTAATTTCTATGCCTGGAAGAG   498
SH   GAGAATATCACTGTCCCAGACACCAAAGTTAATTTCTATGCCTGGAAGAG   497
HE   GAGAATATCACTGTCCCAGACACCAAAGTTAATTTCTATGCCTGGAAGAG   499
     ** ***********************************************

AM   GATGGAGGTGAGTTCCTTTTTTTTTTTTTTTCCTTTCTTTTGGAGAATCT   547
GI   GATGGAGGTGAGTTCCTTTTTTTTTTTTTTTCCTTTCTTTTGGAGAATCT   545
SY   GATGGAGGTGAGTTCCTTTTTTTTTTTTTTTCCTTTCTTTTGGAGAATCT   547
JM   GATGGAGGTGAGTTCCTTTTTTTTTTTTTTTCCTTTCTTTTGGAGAATCT   548
SH   GATGGAGGTGAGTTCCTTTTTTTTTTTTT--TCCTTTCTTTTGGAGAATCT   545
HE   GATGGAGGTGAGTTCCTTTTTTTTTTTTTTTCCTTTCTTTTGGAGAATCT   549
     *****************************  *******************

AM   CATTTGCGAGCCTGATTTTGGATGAAAGGGAGAATGATCGGGGAAAGGT   597
GI   CATTTGCGAGCCTGATTTTGGATGAAAGGGAGAATGATCGAGGAAAGGT   595
SY   CATTTGCGAGCCTGATTTTGGATGAAAGGGAGAATGATCGAGGAAAGGT   597
JM   CATTTGCGAGCCTGATTTTGGATGAAAGGGAGAGTGATCGAGGAAAGGT   598
SH   CATTTGCGAGCCTGATTTTGGATGAAAGGGAGAATGATCGAGGAAAGGT   595
HE   CATTTGCGAGCCTGATTTGGGATGAAAGGGAGAATGATCGAGGAAAGGT   599
     ****************** ************** ****** ********
```

FIG.5D

```
AM    AAAATGGAGCAGCAGAGATGAGGCTGCCTGGGCGCAGAGGCTCACGTCTA   647
GI    AAAATGGAGCAGCAGAGATGAGGCTGCCTGGGCGCAGAGGCTCACGTCTA   645
SY    AAAATGGAGCAGCAGAGATGAGGCTGCCTGGGCGCAGAGGCTCACGTCTA   647
JM    AAAATGGAGCAGCAGAGATGAGGCTGCCTGGGCGCAGAGGCTCACGTCTA   648
SH    AAAATGGAGCAGCAGAGATGAGGCTGCCTGGGCGCAGAGGCTCACGTCTA   645
HE    AAAATGGAGCAGCAGAGATGAGGCTGCCTGGGCGCAGAGGCTCCAGTCTA   649
      *******************************************  *****

AM    TAATCCCAGGCTGAGATGGCCGAGATGGGAGAATTGCTTGAGCCCTGGAG   697
GI    TAATCCCAGGCTGAGATGGCCGAGATGGGAGAATTGCTTGAGCCCTGGAG   695
SY    TAATCCCAGGCTGAGATGGCCGAAATGGGAGAATTGCTTGAGCCCTGGAG   697
JM    TAATCCCAGGCTGAGATGGCCGAGATGGGAGAATTGCTTGAGCCCTGGAG   698
SH    TAATCCCAGGCTGAGACGGCCGAGATGGGAGAATTGCTTGAGCCCTGGAG   695
HE    TAATCCCAGGCTGAGATGGCCGAGATGGGAGAATTGCTTGAGCCCTGGAG   699
      **************** ****** **************************

AM    GTTCAGACCAACCTAGGCAGCATAGTGAGATCCCCCATCTCTACAAACAT   747
GI    GTTCAGACCAACCTAGGCAGCATAGTGAGATCCCCCATCTCTACAAACAT   747
SY    GTTCAGACCAACCTAGGCAGCATAGTGAGATCCCCCATCTCTACAAACAT   747
JM    GTTCAGACCAACCTAGGCAGCATAGTGAGATCCCCCATCTCTACAAACAT   748
SH    GTTCAGACCAACCTAGGCAGCATAGTGAGATCCCCCATCTCTACAAACAT   745
HE    GTTCAGACCAACCTAGGCAGCCTAGTGAGATCCCCCATCTCTACAAACAT   749
      ********************* ****************************

AM    TTAAAAAAATTAGTCAGGTGAAGTGGTGCATGGTGGTAGTCCCAGATATT   797
GI    TTAAAAAAATTAGTCAGGTGAAGTGGTGCATGGTGGTAGTCCCAGATATT   795
SY    TTAAAAAAATTAGTCAGGTGAAGTGGTGCATGGTGGTAGTCCCAGATATT   797
JM    TTAAAAAAATTAGTCAGGTGAAGTGGTGCATGGTGGTAGTCCCAGATATT   798
SH    TTAAAAAAATTAGTCAGGTGAAGTGGTGCATGGTGGTAGTCCCAGATATT   795
HE    TTAAAAAAATTAGTCAGGTGAAGTGGTGCATGGTGGTAGTCCCAGATATT   799
      **************************************************
```

FIG.5E

```
AM    TGGAAGGCTGAGGCGGGAGGATCGCTTGAGCCCAGGAATTTGAGGCTGCA    847
GI    TGGAAGGCTGAGGCGGGAGGATCGCTTGAGCCCAGGAATTTGAGGCTGCA    845
SY    TGGATGGCTGAGGCGGGAGGATCGCTTGAGCCCAGGAATTTGAGGCTGCA    847
JM    TGGAAGGCTGAGGCGGGAGGATCGCTTGAGCCCAGGAATTTGAGGCTGCG    848
SH    TGGAAGGCTGAGGCGGGAGGATCGCTTGAGCCCAGGAATTTGGGGCTGCA    845
HE    TGGAAGGCTGAGGCGGGAGGATCGCTTGAGCCCAGGAATTTGAGGCTGCA    849
      **** ************************************* ****** 

AM    GTGAGCTGTGATCACACCACTGCACTCCAGCCTCAGTGACAGAGTGAGGC    897
GI    GTGAGCTGTGATCACACCACTGCACTCCAGCCTCAGTGACAGAGTGAGGC    895
SY    GTGAGCTGTGATCACACCACTGCACTCCAGCCTCAGTGACAGAATGAGGC    897
JM    GTGAGCTGTGATCACACCACTGCACTCCAGCCTCAGTGACAGAGTGAGGC    898
SH    GTGAGCTGTGATCACACCACTGCAATCCAGCCTCAGTGACAGAGTGAGGC    895
HE    GTGAGCTGTGATCACACCACTGCACTCCAGCCTCAGTGACAGAGTGAGGC    899
      ************************ ****************** ******

AM    CCTGTCTCAAAAAAGAAAAGAAAAAAGAAAAATAATGAGGGCTGTATGGA    947
GI    CCTGTCTCAAAAAAGAAAAGAAAAAAGAAAAATAATGAGGGCTGTATGGA    945
SY    CCTGTCTCAAAAAAGAAAAGAAAAAAGAAAAATAATGAGGGCTGTATGGA    947
JM    CCTGTCTCAAAAAAGAAAAGAAAAAAGAAAAATAATGAGGGCTGTATGGA    948
SH    CCTGTCTCAAAAACGAAAAGAAAAAAGAAAAATAATGAGGGCTGTATGGA    945
HE    CCTGTCTCAAAAAAGAAAAGAAAAAAGAAAAATTATGAGGGCTGTATGGA    949
      ************* ******************* ****************

AM    ATACATTCATTATTCATTCACTCACTCACTCACTCATTTCATTCATTCATT    997
GI    ATACGTTCATTATTCATTCACTCACTCACTCACTCATTTCATTCATTCATT    995
SY    ATACATTCATTATTCATTCACTCACTCACTCACTCATCCATTCATTCATT    997
JM    ATACATTCATTATTCATTCACTCACTCACTCACTCATTTCATTCATTCATT    998
SH    ATACATTCATTATTCATTCACTCACTCACTCACTCATTTCATTCATTCATT    995
HE    ATACATTCATTATTCATTCACTCACTCACTCACTCATTTCATTCATTCATT    999
      **** ******************************** ************
```

FIG.5F

```
AM  CATTCAACAAGTCTTATTGCATACCTTCTGTTTGCTCAGCTTGGTGCTTG  1047
GI  CATTCAACATGTCTTATTGCATACCTTCTGTTTGCTCAGCTTGGTGCTTG  1045
SY  CATTCAACAAGTCTTATTGCATACCTTCTGTTTGCTCAGCTTGGTGCTCG  1047
JM  CATTCAACAAGTCTTATTGCATACCTTCTGTTTGCTCAGCTTGGTGCTTG  1048
SH  CATTCAACAAGTCTTATTGCATACCTTCTGTTTGCTCAGCTTGGTGCTTG  1045
HE  CATTCAACAAGTCTTATTGCATACCTTCTGTTTGCTCAGCTTGGTGCTTG  1049
    ********* ************************************** *

AM  GGGCTGCTGAGGGGCAGGAGGGAGAGGGTGACATGGGTCAGCTGACTCCC  1097
GI  GGGCTGCTGAGGGGCAGGAGGGAGAGGGTGACATCCCTCAGCTGACTCCC  1095
SY  GGGCTGCTGAGGGGCAGGAGGGAGAGGGTGACATGGGTCAGCTGACTCCC  1097
JM  GGGCTGCTGAGGGGCAGGAGGGTGAGGGTGACATGGGTCAGCTGACTCCC  1098
SH  GGCCTTCTGAGGGGCAGGAGGGAGAGGGTGACATGGGTCAGCTGACTCCC  1095
HE  GGGCTGCTGAGGGGCAGGAGGGAGAGGGTGACATGGGTCAACTGACTCCC  1099
    ** ** ***************** ***********   *** *********

AM  AGAGTCCACTCCCTGTAGGTCGGGCAGCAGGCCGTAGAAGTCTGGCAGGG  1147
GI  AGAGTCCACTCCCTGTAGGTCGGGCAGCAGGCCGTAGAAGTCTGGCAGGG  1145
SY  AGAGTCCACTCCCTGTAGGTCGGGCAACAGGCCGTAGAAGTCTGGCAGGG  1147
JM  AGAGTCCACTCCCTGTTGGTCGGGCAGCAGGCCGTAGAAGTCTGGCAGGG  1148
SH  AGAGTCCACTCCCTGTAGGTCGGGCAGCAGGCCGTAGAAGTCTGGCAGGG  1145
HE  AGAGTCCACTCCCTGTAGGTCGGGCAGCAGGCCGTAGAAGTCTGGCAGGG  1149
    **************** ********* ***********************

AM  CCTGGCCCTGCTGTCGGAAGCTGTCCTGCGGGGCCAGGCCCTGTTGGTCA  1197
GI  CCTGGCCCTGCTGTCGGAAGCTGTCCTGCGGGGCCAGGCCCTGTTGGTCA  1195
SY  CCTGGCCCTGCTGTCGGAAGCTGTCCTGCGGGGCCAGGCCCTGTTGGTCA  1197
JM  CCTGGCCCTGCTGTCGGAAGCTGTCCTGCGGGGCCAGGCCCTGTTGGTCA  1198
SH  CCTGGCCCTGCTGTCGGAATCTGTCCTGCGGGGCCAGGCCCTGTTGGTCA  1195
HE  CCTGGCCCTGCTGTCGGAAGCTGTCCTGCGGGGCCAGGCCCTGTTGGTCA  1199
    ******************* ******************************
```

FIG.5G

```
AM   ACTCTTCCCAGCCGTGGGAGCCCCTGCAGCTGCATGTGGATAAAGCCGTC  1247
GI   ACTCTTCCCAGCCGTGGGAGCCCCTGCAGCTGCATGTGGATAAAGCCGTC  1245
SY   ACTTTTCCCAGCCGTGGGAGCCCCTGCAGCTGCATGTGGATAAAGCCGTC  1247
JM   ACTCTTCCCAGCCGTGGGAGCCCCTGCAGCTGCATGTGGATAAAGCCGTC  1248
SH   ACTCTTCCCAACCGTGGGAGCCCCTGCAGCTGCATGTGGATAAAGCCGTC  1245
HE   ACTCTTCCCAGCCGTGGGAGCCCCTGCAGCTGCATGTGGATAAAGCCGTC  1249
     *** ****** ***************************************

AM   AGTGGCCTTCGCAGCCTCACCACTCTGCTTCGGGCTCTGGGAGCCCAGGT  1297
GI   AGTGGCCTTCGCAGCCTCACCACTCTGCTTCGGGCTCTGGGAGCCCAGGT  1295
SY   AGTGGCCTTCGCAGCCTCACCACTCTGCTTCGGGCTCTGGGAGCCCAGGT  1297
JM   AGTGGCCTTCGCAGCCTCACCACTCTGCTTCGGGCTCTGGGAGCCCAGGT  1298
SH   AGTGGCCTTCGCAGCCTCACCACTCTGCTTCGGGCTCTGGGAGCCCAGTT  1295
HE   AGTGGCCTTCGCAGCCTCACCACTCTGCTTCGGGCTCTGGGAGCCCAGGT  1299
     ************************************************ *

AM   GAGTAGGAGCGGACACTTCTGCTTGCCCTTTCTGTAAGAAGGGGAGAAGG  1347
GI   GAGTAGGAGCGGACACTTCTGCTTGCCCTTTCTGTAAGAAGGGGAGAAGG  1345
SY   GAGTAGGAGCGGACACTTCTGCTTGCCCTTTCTGTAAGAAGGGGAGAAGG  1347
JM   GAGTAGGAGCGGACACTTCTGCTTGCCCTTTCTGTAAGAAGGGGAGAAGG  1348
SH   GAGTAGGAGGGGACACTTCTGCTTGCCCTTTGTGTAAGAAGGAGAGAAGG  1345
HE   GAGTAGGAGCGGACACTTCTGCTTGCCCTTTCTGTAAGAAGGGGAGAAGG  1349
     ********* ********************* ********** *******

AM   GTCTTGCTAAGGAGTACAGGAACTGTCCGTATTCCTTCCCTTTCTGTGGC  1397
GI   GTCTTGCTAAGGAGTACAGGAACTGTCCGTATTCCTTCCCTTTCTGTGGC  1395
SY   GTCTTGCTAAGGAGTACAGGAACTGTCCGTATTCCTTCCCTTTCTGTGGC  1397
JM   GTCTTGCTAAGGAGTACAGGAACTGTCCGTATTCCTTCCCTTTCTGTGGC  1398
SH   GTCTTGCTAAGGAGTACAGGAACTGTCCGTATTCCTTCCCTTTCTGTGGC  1395
HE   GTCTTGCTAAGGAGTACAGGATCTGTCCGTATTCCTTCCCTTTCTGTGGC  1399
     ********************* ****************************
```

FIG.5H

```
AM    ACTGCAGCGACCTCCTGTTTTCTCCTTGGCAGAAGGAAGCCATCTCCCCT  1447
GI    ACTGCAGCGACCTCCTGTTTTCTCCTTGGCAGAAGGAAGCCATCTCCCCT  1445
SY    ACTGCAGCGACCTCCTGTTTTCTCCTTGGCAGAAGGAAGCCATCTCCCCT  1447
JM    ACTGCAGCGACCTCCTGTTTTCTCCTTGGCAGAAGGAAGCCATCTCCCCT  1448
SH    ACTGCAGCGACCTCCTGTTTTCTCCTTGGCAGAAGGAAGCCATCTCCCCT  1445
HE    ACTGCAGCGACCACCTGTTTTCTCCTTGGCAGAAGGAAGCCATCTCCCCT  1449
      ************ *************************************

AM    CCAGATGCGGCCTCAGCTGCTCCACTCCGAACAATCACTGCTGACACTTT  1497
GI    CCAGATGCGGCCTCAGCTGCTCCACTCCGAACAATCACTGCTGACACTTT  1495
SY    CCAGATGCGGCCTCAGCTGCTCCACTCCGAACAATCACTGCTGACACTTT  1497
JM    CCAGATGCGGCCTCAGCTGCTCCACTCCGAACAATCACTGCTGACACTTT  1498
SH    CCAGATGCGGCCTCAGCTGCTCCACTCCGAACAATCACTGCTGATACTTT  1495
HE    CCAGATGCGGCCTCAGCTGCTCCACTCCGAACAATCACTGCTGACACTTT  1499
      ********************************************* *****

AM    CCGCAAACTCTTCCGAGTCTACTCCAATTTCCTCCGGGGAAAGCTGAAGC  1547
GI    CCGCAAACTCTTCCGAGTCTACTCCAATTTCCTCCGGGGAAAGCTGAAGC  1545
SY    CCGCAAACTCTTCCGAGTCTACTCCAATTTCCTCCGGGGAAAGCTGAAGC  1547
JM    CCGCAAACTCTTCCGAGTCTACTCCAATTTCCTCCGGGGAAAGCTGAAGC  1548
SH    CCGCAAACTCTTCCGAGTCTACTCCAATTTCCTCCGGGGAAAGCTGAAGC  1545
HE    CCGCAAACTCTTCCGAGTCTACTCCAATTTCCTCCGGGGAGAGCTGAAGC  1549
      **************************************** *********

AM    TGTACACAGGGGAGGCCTGCAGGACAGGGGACAGATGA  1584
GI    TGTACACAGGGGAGGCCTGCAGGACAGGGGACAGATGA  1582
SY    TGTACACAGGGGAGGCCTGCAGGACAGGGGACAGATGA  1585
JM    TGTACACAGGGGAGGCCTGCAGGACAGGGGACAGATGA  1585
SH    TGTACACAGGGGAGGCCTGCAGGACAGGGGACAGATGA  1583
HE    TGTACACAGGGGAGGCCTGCAGGACAGGGGACGGATGA  1586
      ******************************** *****
```

FIG.6

```
AM/GI   MGVHECPAWLWLLLSLLSLPLGLPVLGAPPRLICDSRVLERYLLEAKEAE    50
SY      MGVHECPAWLWLLLSLLSLPLGLPVLGAPPRLICDSRVLERYLLEAKEAE    50
JM      MGVHECPAWLWLLLSLLSLPLGLPVLGAPPRLICDSRVLERYLLEAKEAE    50
SH      MGVHECPAWLWLLLSLLSLPLGLPVLGAPPRLICDRRVLERYLLEAKEAE    50
HE      MGVHECPAWLWLLLSLLSLPLGLPVLGAPPRLICDSRVLERYLLEAKEAE    50
        *********************************** **************

AM/GI   NITTGCAEHCSLNENITVPDTKVNFYAWKRMEVGQQAVEVWQGLALLSEA   100
SY      NITTGCAEHCSLNENITVPDTKVNFYAWKRMEVGQQAVEVWQGLALLSEA   100
JM      NITKGCAEHCSLNENITVPDTKVNFYAWKRMEVGQQAVEVWQGLALLSEA   100
SH      NITTGCAEHCSLNENITVPDTKVNFYAWKRMEVGQQAVEVWQGLALLSES   100
HE      NITTGCAEHCSLNENITVPDTKVNFYAWKRMEVGQQAVEVWQGLALLSEA   100
        *** **********************************************

AM/GI   VLRGQALLVNSSQPWEPLQLHVDKAVSGLRSLTTLLRALGAQKEAISPPD   150
SY      VLRGQALLVNSSQPWEPLQLHVDKAVSGLRSLTTLLRALGAQKEAISPPD   150
JM      VLRGQALLVNSSQPWEPLQLHVDKAVSGLRSLTTLLRALGAQKEAISPPD   150
SH      VLRGQALLVNSSQPWEPLQLHVDKAVSGLRSLTTLLRALGAQKEAISPPD   150
HE      VLRGQALLVNSSQPWEPLQLHVDKAVSGLRSLTTLLRALGAQKEAISPPD   150
        **************************************************

AM/GI   AASAAPLRTITADTFRKLFRVVSNFLRGKLKLYTGEACRTGDR   193
SY      AASAAPLRTITADTFRKLFRVVSNFLRGKLKLYTGEACRTGDR   193
JM      AASAAPLRTITADTFRKLFRVYSNFLRGKLKLYTGEACRTGDR   193
SH      AASAAPLRTITADTFRKLFRVYSNFLRGKLKLYTGEACRTGDR   193
HE      AASAAPLRTITADTFRKLFRVYSNFLRGELKLYTGEACRTGDG   193
        ********************* ****** ************* 
```

FIG.7

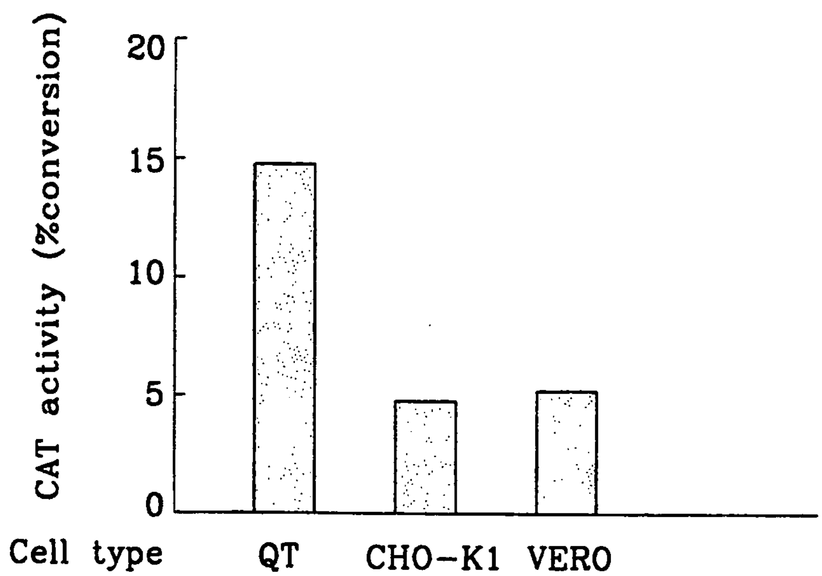


FIG.8

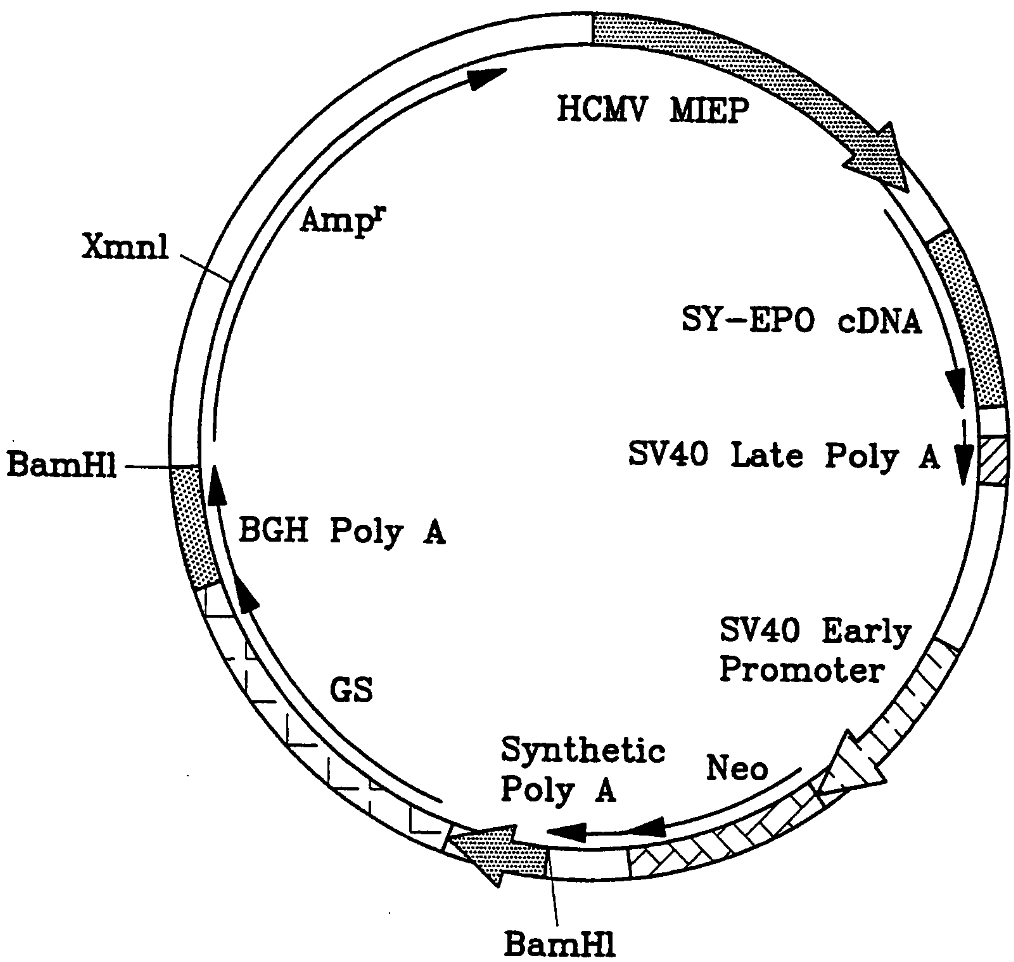

HCMV MIEP
Amp^r
XmnI
SY-EPO cDNA
SV40 Late Poly A
BamHI
BGH Poly A
SV40 Early
Promoter
GS
Synthetic
Poly A
Neo
BamHI

FIG.9

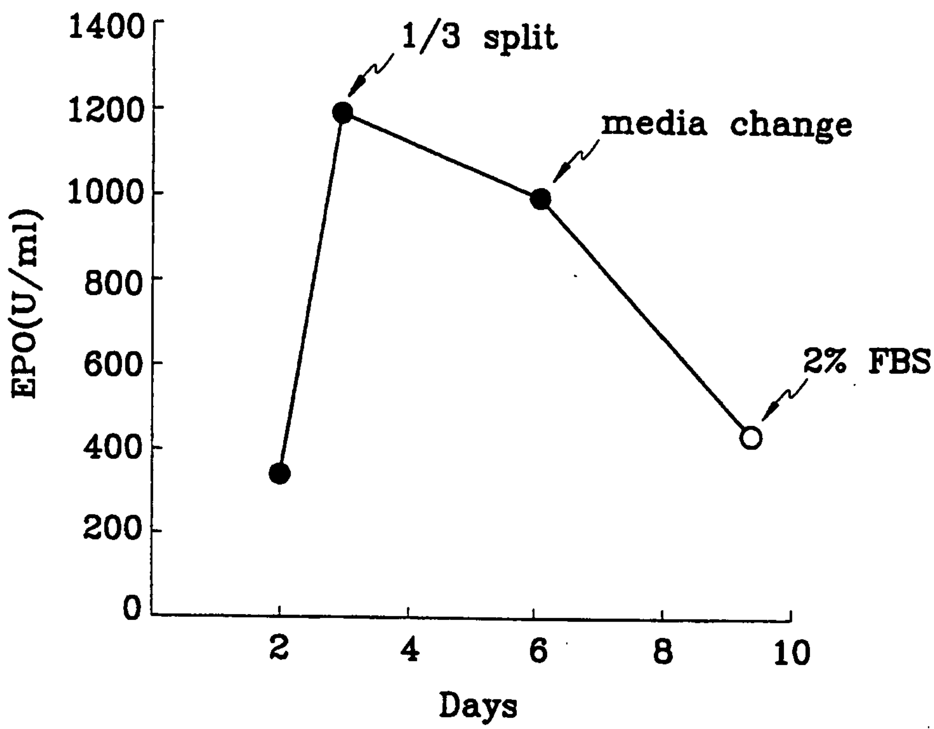
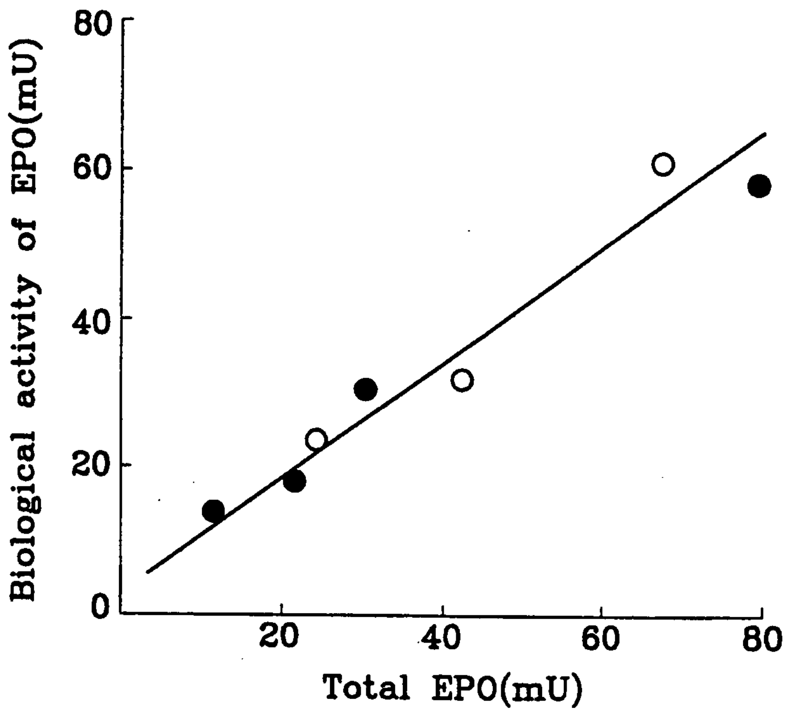

1400
1200
1000
800
600
400
200
0
EPO(U/ml)
1/3 split
media change
2% FBS
2
4
6
8
10
Days

FIG.10


Biological activity of EPO(mU)
80
60
40
20
0
20
40
60
80
Total EPO(mU)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 96/00145

A. CLASSIFICATION OF SUBJECT MATTER

IPC^6: C 12 N 15/11, 15/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC^6: C 12 N 15/11, 15/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

ie

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 89/ 03 877 A1 (INSTITUT INTERNATIONAL DE LA RECHERCHE AGRONOMIQUE (INRA)) 05 May 1989 (05.05.89), claims 1,18,21,23. -----	1,8

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 November 1996 (08.11.96)	05 December 1996 (05.12.96)

Name and mailing address of the ISA/ AT	Authorized officer
AUSTRIAN PATENT OFFICE Kohlmarkt 8-10 A-1014 Vienna Facsimile No. 1/53424/535	Wolf Telephone No. 1/53424/133

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/KR 96/00145

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
WO A1 8903877	05-05-89	EP A1 395658	07-11-90
		FR A1 2622207	26-04-89
		FR B1 2622207	09-03-90
		JP T2 3503839	29-08-91
		US A 5252465	12-10-93

JP006867

WO9810650[1].pdf
06/27/08 09:42 PM

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A01N 37/18, A61K 38/00

A1

(11) International Publication Number: WO 98/10650

(43) International Publication Date: 19 March 1998 (19.03.98)

(21) International Application Number: PCT/US97/15966

(22) International Filing Date: 10 September 1997 (10.09.97)

(30) Priority Data:
08/712,358 11 September 1996 (11.09.96) US

(71) Applicant: EAST CAROLINA UNIVERSITY [US/US]; 210 Spilman Building, Greenville, NC 27858-4353 (US).

(71)(72) Applicants and Inventors: ANAGNOSTOU, Athanasius, A. [US/US]; 110 Kenilworth Road, Greenville, NC 27858 (US). SIGOUNAS, George [GR/US]; 313 Queen Agnes Road, Greenville, NC 27858 (US).

(74) Agents: BENNETT, Virginia, C. et al.; Myers Bigel Sibley & Sajovec, P.O. Box 37428, Raleigh, NC 27627 (US).

(81) Designated States: CA, CN, JP, MX, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: METHOD OF TREATING ENDOTHELIAL INJURY

(57) Abstract

The use of human erythropoietin (EPO) to prevent or treat endothelial injury due to chemotherapy, radiation therapy, mechanical trauma, or to a disease state which damages the endothelium (such as inflammation, heart disease or cancer) is described. The use of EPO in conjuction with the administration of chemotherapeutic agents is described.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

METHOD OF TREATING ENDOTHELIAL INJURY

Field of the Invention

The present invention relates to the use of human erythropoietin (EPO) in the prevention or treatment of endothelial injury due to chemotherapy, radiation therapy, mechanical trauma, or to a disease state which damages the endothelium (such as inflammation, heart disease or cancer). The present invention further relates to the use of EPO in conjunction with chemotherapy.

Background of the Invention

Erythropoietin (EPO) is a glycoprotein produced in the kidney, and is the principal hormone responsible for stimulating red blood cell production (erythrogenesis). EPO stimulates the division and differentiation of committed erythroid progenitors in the bone marrow. Normal plasma erythropoietin levels range from 0.01 to 0.03 Units/mL, and can increase up to 100 to 1,000-fold during hypoxia or anemia. Graber and Krantz, *Ann. Rev. Med.* 29:51 (1978); Eschbach and Adamson, *Kidney Intl.* 28:1 (1985). Recombinant human erythropoietin (rHuEpo or epoetin alfa) is commercially available as Epogen® (Amgen Inc., Thousand Oaks, CA) and as Procrit® (Ortho Biotech Inc., Raritan, NJ). EPO is indicated for treatment of anemia, including anemias associated with cancer chemotherapy, chronic renal failure, malignancies,

adult and juvenile rheumatoid arthritis, disorders of haemoglobin synthesis, prematurity, and zidovudine treatment of HIV infection.

The vascular endothelium is a layer of cells lining the inner vascular wall and in direct contact with blood, providing an active natural barrier between the circulatory and extravascular compartment. The endothelium is involved in signal and information transfer at the cellular, tissue and organ level, and plays a role in both cell-mediated and humoral immune responses. Endothelial cells are metabolically active and normally produce a number of substances with effects on the vascular lumen and on platelets. Endothelial vasodilators include prostacyclin (PGI_2) and endothelium-derived relaxing factor (EDRF, which may be nitric oxide or a more stable adduct thereof); these two substances also act to inhibit platelet aggregation.

Damage or destruction of the endothelium by physical trauma or disease processes such as atherosclerotic plaque formation may impair EDRF production, contributing to vasoconstriction. More diffuse and subtle endothelial damage, such as due to chronic hypertension or reperfusion after ischemia, also leads to altered EDRF production. Endothelial products localized to the luminal endothelial surface include ectoADPase and thrombomodulin. Vasoconstrictors released by the endothelium include endothelin. Endothelial cells also secrete growth factors which enhance endothelial mitogenesis and can induce new blood vessel formation (angiogenesis). It has been reported that granulocyte macrophage-colony stimulating factor (GM-CSF) and granulocyte-colony stimulating factor (G-CSF) stimulate proliferation and migration of endothelial cells. Interleukin-3 (IL-3) also enhances the proliferation of these cells. *See* Bussolino et al., *Nature* 337:471 (1989); Brizzi et al., *J. Clin. Invest.* 91:2887 (1993).

Summary of the Invention

A first aspect of the present invention is a method of reducing endothelial injury caused by a chemotherapeutic agent, by administering an endothelial-protecting amount of erythropoietin in conjunction with the administration of the chemotherapeutic agent. The endothelial-protecting amount of erythropoietin may be administered simultaneously with the chemotherapeutic agent, prior to the chemotherapeutic agent, or after the chemotherapeutic agent.

A second aspect of the present invention is a method of enhancing endothelial cell inhibition in a subject treated with a chemotherapeutic agent, by administering an endothelial-inhibiting amount of erythropoietin in conjunction with the chemotherapeutic agent. The endothelial-inhibiting amount of erythropoietin may be administered simultaneously with, prior to, or after the chemotherapeutic agent.

A further aspect of the present invention is a method of treating a solid vascularized tumor by administering an antineoplastic chemotherapeutic agent in conjunction with an endothelial-inhibiting amount of erythropoietin. The endothelial-inhibiting amount of erythropoietin may be administered simultaneously with, prior to, or after the chemotherapeutic agent.

A further aspect of the present invention is a method of treating endothelial injury caused by mechanical damage, exposure to radiation, inflammation, heart disease or cancer by administering an endothelial-protecting amount of erythropoietin to a subject in need of such treatment.

The foregoing and other objects and aspects of the present invention are explained in detail in the specification set forth below.

Brief Description of the Drawings

Figure 1 is a graph showing the dose-response curve for viability of endothelial cells after exposure to cisplatin.

Figure 2 is a graph showing the responses of endothelial cell cultures exposed simultaneously to cisplatin and varying dosages of EPO, compared to control endothelial cell cultures exposed only to cisplatin.

Figure 3 is a graph showing the responses of endothelial cell cultures exposed first to cisplatin and, two hours later, to varying dosages of EPO (compared to control endothelial cell cultures exposed only to cisplatin).

Figure 4 is a graph showing the responses of endothelial cell cultures exposed first to varying dosages of EPO and, two hours later, to cisplatin (compared to control endothelial cell culture exposed only to cisplatin).

Detailed Description of the Invention

The present inventors have previously shown that recombinant human erythropoietin (EPO) exerts a mitogenic and chemoattractant (migratory) effect on human umbilical vein endothelial cells and bovine capillary endothelial cells. Anagnostou et al., *Proc. Natl. Acad. Sci. USA* 87:5978 (1990). Endothelial cell migration and proliferation are the key steps in the angiogenic process.

The present inventors have found that EPO can effectively prevent and/or repair endothelial damage caused by chemotherapeutic agents. The present inventors have found that administration of EPO concomitantly with chemotherapeutic agents produces a biphasic response: certain doses of EPO protect endothelial cells from the deleterious effects of the chemotherapeutic agent, while

increased doses enhance the endothelial growth-suppression caused by the chemotherapeutic agent.

The use of EPO to enhance endothelial growth-suppression during chemotherapy is useful in treating angiogenic tumors, where it is desirable to prevent or slow the formation of new blood vessels which support tumor growth. Tumors require an adequate blood supply, and growth of new vessels in the tumor mass is stimulated by angiogenic factors secreted by tumor tissue. In animal models, inhibition of angiogenesis in tumor tissue has been shown to cause tumor regression. Highly vascularized solid tumors include cerebellar hemangioblastoma, ductal carcinoma of the breast, and squamous cell cancer of the larynx. Abnormal angiogenesis is involved in additional pathological conditions, including diabetic retinopathy, neovascular glaucoma, rheumatoid arthritis, and psoriasis. The ability of EPO to reduce or prevent abnormal angiogenesis will be of use in preventing or reducing angiogenesis associated with such disease states.

One method according to the present invention is the use of EPO as an adjunct in the chemotherapy of neoplastic disease. EPO is provided in endothelial-protecting amounts where protection of the endothelium from the adverse effects of chemotherapeutic agents is desired. A second method according to the present invention is the use of EPO as an adjunct in the chemotherapy of neoplastic disease, where enhancement of the adverse effects of chemotherapeutic agents on endothelium (e.g., enhancement of endothelial growth suppression) is desired. In such situations, EPO is provided in endothelial-inhibiting amounts.

As used herein, endothelial-protecting amounts of EPO refer to those dosages which reduce or prevent the suppression of endothelial growth which would otherwise occur due to exposure to a chemotherapeutic agent or radiation, mechanical trauma, or a disease state known to

damage the endothelium. Alternatively, an endothelial-protecting amount of EPO may be defined as those dosages which increase the numbers of viable endothelial cells following exposure to the chemotherapeutic agent or radiation, mechanical trauma, or a disease state known to damage the endothelium; the increased number of viable cells is in comparison to that which would be expected in the absence of EPO. The most effective endothelial-protecting amounts of EPO may vary depending upon the time of administration and the etiology of endothelial damage.

Where endothelial damage is due to exposure to a chemotherapeutic agent, the most effective endothelial-protecting amounts of EPO will vary depending upon whether EPO is administered simultaneously with, prior to, or after, the chemotherapeutic agent, and may vary depending upon the specific chemotherapeutic agent in question.

As used herein, endothelial-inhibiting amounts of EPO refer to those dosages which enhance or increase the suppression of endothelial growth which would otherwise occur due to exposure to a chemotherapeutic agent or radiation, mechanical trauma, or a disease state known to damage the endothelium. Alternatively, an endothelial-inhibiting amount of EPO may be defined as those dosages which decrease the numbers of viable endothelial cells following exposure to the chemotherapeutic agent or radiation, mechanical trauma, or a disease state known to damage the endothelium; the decreased number of viable cells is in comparison to that which would be expected in the absence of EPO. The most effective endothelial-inhibiting amounts of EPO may vary depending upon the time of administration and the etiology of endothelial damage.

Where endothelial damage is due to exposure to a chemotherapeutic agent, the most effective inhibiting amounts of EPO will vary depending upon whether EPO is

administered simultaneously with, prior to, or after, the chemotherapeutic agent, and may vary depending upon the specific chemotherapeutic agent in question.

Endothelial damage may be assessed by a reduction in the proliferation of endothelial cells and/or decreased numbers of viable endothelial cells, leading to a total decrease in the number of viable endothelial cells. Such a decrease in the number of viable endothelial cells may also be referred to as endothelial growth suppression, or endothelial cell suppression or inhibition.

As used herein, a method of reducing endothelial injury in a subject caused by administration of a chemotherapeutic agent to the subject refers to a method which reduces or prevents the decrease in viable endothelial cells which would otherwise be caused by administration of the chemotherapeutic agent. As used herein, a method of enhancing endothelial cell inhibition in a subject caused by administration of a chemotherapeutic agent to the subject refers to a method which increases or enhances the reduction in viable endothelial cells which would otherwise be caused by administration of the chemotherapeutic agent.

Damage to endothelial cells may also be caused by radiation therapy, mechanical trauma, and by disease states such as inflammation, heart disease (*e.g.*, atherosclerosis) and cancer. In atherosclerosis, for example, injury to or dysfunction of the endothelium leads to reduced vasodilator response and to increased platelet deposition on the arterial wall. Serotonin and thromboxane A_2 released from deposited platelets cause arterial constriction and spasm, increase adhesion and aggregation of platelets, and enhance the atherosclerotic process. The consequences of coronary obstruction are often ameliorated by the formation of new coronary vessels in response to angiogenic stimuli. The use of EPO to enhance endothelial growth and/or repair, or to

prevent endothelial damage, will be a useful adjunct in treating endothelial damage due to mechanical damage, radiation therapy, or due to disease states which adversely affect the endothelium.

As used herein, human erythropoietin (EPO) refers to both the naturally occurring human erythropoietin glycoprotein as well as recombinant human erythropoietin (rHuEpo or epoetin alfa, available commercially as Epogen® (Amgen Inc., Thousand Oaks, CA) and as Procrit® (Ortho Biotech Inc., Raritan, NJ)). Peptide analogs of EPO may also be used in the methods of the present invention. As used herein, peptide analogs are those compounds which, while not having amino acid sequences identical to that of EPO, have a similar three-dimensional structure. In protein molecules which interact with a receptor, the interaction takes place at the surface-accessible sites in a stable three-dimensional molecule. By arranging the critical binding site residues in an appropriate conformation, peptides which mimic the essential surface features of EPO binding region may be designed and synthesized in accordance with known techniques. A molecule which has a surface region with essentially the same molecular topology to the binding surface of EPO will be able to mimic the interaction of EPO with the EPO receptor. Methods for determining peptide three-dimensional structure and analogs thereto are known, and are sometimes called 'rational drug design techniques'. *See, e.g.*, U.S. Patent No. 4,833,092 to Geysen; U.S. Patent No. 4,859,765 to Nestor; U.S. Patent No. 4,853,871 to Pantoliano; U.S. Patent No. 4,863,857 to Blalock (applicants specifically intend that the disclosures of all U.S. patents cited herein be incorporated by reference in their entirety).

Peptides which mimic the biological activity of erythropoietin (EPO receptor peptide ligands) may be substituted for EPO in the methods of the present invention. The sequence of such peptides may represent

fragments of the full-length EPO protein sequence, which fragments are capable of binding to and activating the EPO receptor. Additionally, peptides with sequences dissimilar to that of EPO may be utilized in the methods of the present invention, where such peptides mimic the biological activity of EPO. Wrighton et al. report the identification and characterization of small peptides that bind to and activate the erythropoietin receptor on the surface of target cells, although the peptides' sequences are not similar to the primary sequence of EPO (Wrighton et al., *Science* 273:458 (26 July 1996)). These peptide agonists are represented by a 14-amino acid disulfide-bonded cyclic peptide with an identified minimum consensus sequence. The structure of a complex of one such peptide mimetic with the erythropoietin receptor is described by Livnah et al., *Science* 273:464 (26 July 1996).

As used herein, the term chemotherapeutic agent refers to cytotoxic antineoplastic agents, that is, chemical agents which preferentially kill neoplastic cells or disrupt the cell cycle of rapidly proliferating cells, used therapeutically to prevent or reduce the growth of neoplastic cells. Chemotherapeutic agents are also known as antineoplastic drugs or cytotoxic agents, and are well known in the art. As used herein, chemotherapy includes treatment with a single chemotherapeutic agent or with a combination of agents. In a subject in need of treatment, chemotherapy may be combined with surgical treatment or radiation therapy, or with other antineoplastic treatment modalities.

Exemplary chemotherapeutic agents are vinca alkaloids, epipodophyllotoxins, anthracycline antibiotics, actinomycin D, plicamycin, puromycin, gramicidin D, paclitaxel (Taxol®, Bristol Myers Squibb), colchicine, cytochalasin B, emetine, maytansine, and amsacrine (or "mAMSA"). The vinca alkaloid class is described in <u>Goodman and Gilman's The Pharmacological</u>

Basis of Therapeutics, 1277-1280 (7th ed. 1985) (hereafter "Goodman and Gilman"). Exemplary of vinca alkaloids are vincristine, vinblastine, and vindesine. The epipodophyllotoxin class is described in Goodman and Gilman, supra at 1280-1281. Exemplary of epipodophyllotoxins are etoposide, etoposide orthoquinone, and teniposide. The anthracycline antibiotic class is described in Goodman and Gilman, supra at 1283-1285. Exemplary of anthracycline antibiotics are daunorubicin, doxorubicin, mitoxantraone, and bisanthrene. Actinomycin D, also called Dactinomycin, is described in Goodman and Gilman, supra at 1281-1283. Plicamycin, also called mithramycin, is described in Goodman and Gilman, supra at 1287-1288. Additional chemotherapeutic agents include cisplatin (Platinol®, Bristol Myers Squibb); carboplatin (Paraplatin®, Bristol Myers Squibb); mitomycin (Mutamycin®, Bristol Myers Squibb); altretamine (Hexalen®, U.S. Bioscience, Inc.); cyclophosphamide (Cytoxan®, Bristol Myers Squibb); lomustine [CCNU] (CeeNU®, Bristol Myers Squibb); carmustine [BCNU] (BiCNU®, Bristol Myers Squibb).

Methods of administering chemotherapeutic drugs vary depending upon the specific agent used, as would be known to one skilled in the art. Depending upon the agent used, chemotherapeutic agents may be administered, for example, by injection (intravenously, intramuscularly, intraperitoneally, subcutaneously, intratumor, intrapleural) or orally.

As used herein, the administration of a compound "in conjunction with" a second compound means that the two compounds are administered closely enough in time that the presence of one alters the biological effects of the other. The two compounds may be administered simultaneously (concurrently) or sequentially. Simultaneous administration may be carried out by mixing the compounds prior to administration, or

by administering the compounds at the same point in time but at different anatomic sites or using different routes of administration.

The phrases "concurrent administration", "simultaneous administration" or "administered simultaneously" as used herein, means that the compounds are administered at the same point in time or immediately following one another. In the latter case, the two compounds are administered at times sufficiently close that the results observed are indistinguishable from those achieved when the compounds are administered at the same point in time.

Subjects to be treated by the method of the present invention include both human and animal (e.g., dog, cat, cow, horse) subjects, and are preferably mammalian subjects.

Many chemotherapeutic agents act at specific phases of the cell cycle, and are active only against cells in the process of division. Neoplasms which are the most susceptible to chemotherapy are those with a high percentage of cells in the process of division, including but not limited to breast, liver, brain, lung, and ovarian cancer. Highly vascularized solid tumors are amenable to treatment with endothelial-inhibiting amounts of EPO in conjunction with chemotherapeutic agents, as these tumors rely on angiogenesis to provide adequate blood supply to the growing tumor tissue.

EPO used according to the methods of the present invention may be administered by any suitable means, as would be apparent to one skilled in the art. EPO may be administered systemically (e.g., intravenously) or locally (e.g., injected into a tumor, tissues immediately surrounding a tumor, or into an anatomic compartment containing a tumor). For example, where an endothelial-inhibiting amount of EPO is utilized as an adjunct to chemotherapy, the EPO may be administered locally to a tumor (or the immediately

surrounding tissue) in which it is desirable to prevent angiogenesis. Where a chemotherapeutic agent is delivered systemically, for example, an endothelial-protecting amount of EPO may be administered systemically by intravenous injection.

The dosage and timing of EPO administration used in conjunction with a chemotherapeutic agent will similarly depend upon the desired effect. The present inventors have discovered that depending upon the timing of EPO administration (simultaneous with, before, or after chemotherapeutic agent administration) and the dosage of EPO, EPO either protects the endothelium from the growth-inhibiting effects of chemotherapeutic agents, or enhances the endothelial growth inhibition seen with chemotherapeutic agents. It will be apparent to those skilled in the art how to determine, by routine experimentation, the dosage and timing of EPO administration in conjunction with a particular chemotherapeutic agent to achieve a desired effect.

The maximum amount of EPO that can be administered in single or multiple doses has not been determined. Doses of up to 1,500 Units/kg for three to four weeks have been administered without toxic effects due to EPO itself. Eschbach et al., in: <u>Prevention of Chronic Uremia</u> (Friedman et al., eds.), Field and Wood Inc., Philadelphia, pp. 148-155 (1989). In the present methods, where it is desired to protect the endothelium from the endothelial damage and/or endothelial growth suppression caused by a chemotherapeutic agent, EPO is administered in an endothelial-protecting amount. Suitable endothelial-protecting dosages may range from about 100 U/kg to about 200 U/kg. In the present methods, where it is desired to enhance the endothelial damage and/or endothelial growth suppression caused by a chemotherapeutic agent, EPO is administered in an endothelial-inhibiting amount which may range from about 750 U/kg to about 2,000 U/kg. As noted above, the

dosage and timing of EPO administration used in conjunction with a chemotherapeutic agent will depend upon the desired effect, as well as the chemotherapeutic agent utilized.

The following examples are provided to illustrate the present invention, and should not be construed as limiting thereof.

EXAMPLE 1

Materials and Methods

Cell Culture. Human umbilical vein endothelial cells (HUVECs) were obtained from Caesarian section derived cords. HUVECs were cultured by standard methodology in 25 cm^2 T-flasks (Corning Inc., Corning, NY) coated with 0.5% porcine skin gelatin (Sigma Chemical Co., St. Louis, MO). Medium 199 (Life Technologies, Gaithersburg, MD), supplemented with 20% defined fetal bovine serum (FBS) (Hyclone, Logan, UT), 16 U/ml heparin (Sigma), 50 μg/ml bovine hypothalamus derived endothelial mitogen (Biomedical Technologies, Stoughton, MA), 100 U/ml penicillin and 100 μg/ml streptomycin was used for the growth of HUVECs. Endothelial cells were characterized by homogenous and typical cobblestone morphology, von Willebrand factor antigen positivity, and the presence of Weibel-Palade bodies, as are known in the art.

Protection/Inhibition Assay. The number of metabolically active cells after exposure of endothelial cell cultures to test agents was assessed using a colorimetric method. This assay utilizes solutions of a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4 Sulfophenyl)-2H-tetrazolium] (MTS) and an electron coupling reagent, phenazine methosulfate (PMS; available from Promega Corp., Madison, Wisconsin). *See* Denizot and Lang, *J. Immunol. Methods* 89:271 (1986); Promega Corporation Technical Bulletins

112, 152 and 169). MTS is bioreduced into a formazan by dehydrogenase enzymes found in metabolically active cells. The amount of formazan is measured at 490 nm absorbance and is directly proportional to the numbers of living cells in culture.

Endothelial cells grown in the complete (supplemented) M199 medium were harvested in the log phase. At 80-90% confluency, EC culture monolayers were washed with phosphate buffered saline (PBS), treated with 0.25% trypsin in 1 mM EDTA for 1-2 minutes, and then the cells were suspended in complete medium. The number and viability of the cells was determined using a hemocytometer and the trypan blue staining, respectively. Cell suspensions of 7.22 x 10^4 cells/ml medium were prepared and 90μl (6.5 x 10^3 cells) were dispensed into each well of a 96-well plate. After overnight incubation at 37°C, 5% CO_2, in a humidified atmosphere, EPO and/or the chemotherapeutic agent were added at concentrations and in the order specified in the examples described below. Plates were then incubated for another 24 hours. At the end of this incubation period, 20 μl of freshly prepared combined MTS/PMS (20:1 ratio) solution was added into each well and the plates were incubated for 1-4 more hours, as per manufacturer's recommendations. The absorbance of each well at 490 nm was recorded using an ELISA plate reader. The LD50 and the effect of the various treatments on cell viability and chemosensitivity were determined by plotting the corrected absorbance at 490 nm versus the concentration of the additive (EPO, chemotherapeutic agent, or combinations thereof).

<u>Statistical Considerations</u>. For protection/inhibition assays, experiments were performed in triplicate. All other experiments were performed at least five times. Results were averaged and means ± SD reported. Controls for all experiments included one to two triplicate wells treated with each of the following:

1) 1 μg/ml cisplatin;

2) 50 μg/ml cisplatin;
3) 10 or 20 U/ml of EPO;
4) 0.6 or 1.2 U/ml of EPO.

Thus for each experiment, three to six wells received the above four control treatments (total 12-24 control wells). An additional control consisting of a triplicate well of untreated cells was also performed.

EXAMPLE 2

Determination of Cisplatin LD50

Ninety-six-well plates containing endothelial cells were prepared as described in Example 1 and incubated overnight at 37°C, 5% CO_2, in a humidified atmosphere. A solution of 160 μg/ml cisplatin was prepared, and serial dilutions were added to the wells (5 μl per well; concentrations varied from 0.03125 μg/ml to 4.0 μg/ml. The plates were then incubated for two days (48 hours) and viability of endothelial cells was assessed using the MTS/PMS technique described in Example 1. The absorbance of each well at 490 nm was recorded using an ELISA plate reader. The corrected absorbance at 490 nm versus the concentration of cisplatin (μg/mL) was plotted (**Figure 1**) to provide a dose-response curve. The concentration of cisplatin required to give 50% of the maximal response (LD50 of cisplatin) was determined to be 0.45 μg/ml.

In view of the above findings, a dosage of 1 μg/ml of cisplatin was used to determine the effects of EPO on endothelial cells, as provided in the following examples.

EXAMPLE 3

Effects of Simultaneous Cisplatin and EPO on Endothelial Cells

To determine the effects of combined EPO and cisplatin on endothelial cells, serial dilutions of EPO

were added to endothelial cell cultures simultaneously with cisplatin.

Endothelial cell cultures were prepared as described in Example 1. Cisplatin (final concentration of 1 μg/ml) was added to each test well simultaneously with 5 μl of various EPO preparations (final EPO concentration ranging from 0.15 to 20 U/ml. Endothelial cell viability was assessed using the MTS/PMS colorimetric assay described in Example 1. Results were compared to control wells (endothelial cells treated with 1 μg/ml cisplatin alone, considered as the baseline and represented in Figure 2 as 0%). Results are provided in Figure 2; the "% of control" is the percentage change of optical density at 490 nm over the control, such that "0%" indicates the test well had similar numbers of metabolically active cells as the control, whereas "50%" indicates 50% more and "-50%" indicates 50% fewer metabolically active cells.

As shown in **Figure 2**, a biphasic response was observed when EPO was added to cell cultures simultaneously with the addition of cisplatin. Endothelial cell cultures treated with from 0.15 to 1.25 U/ml of EPO were protected from the damaging effects of cisplatin when EPO was added simultaneously with cisplatin. EPO concentrations of 0.3 U/ml provided the greatest protection of endothelial cells when EPO was added simultaneously with cisplatin; the number of viable cells was approximately 30% greater than that observed in control cultures treated with cisplatin only.

As also shown in **Figure 2**, endothelial cell growth was inhibited in cultures treated with from 5 to 20 U/ml of EPO when EPO was added simultaneously with cisplatin, compared to cultures treated with cisplatin alone. Cultures treated with 5 U/ml of EPO and 1 μg/ml cisplatin showed a 33% decrease in the number of viable cells compared to control cells exposed to cisplatin alone.

EXAMPLE 4

Effects of EPO on Endothelial Cells Administered After Cisplatin Exposure

In this experiment, serial dilutions of EPO were added to endothelial cell cultures two hours after the cultures were exposed to cisplatin.

Endothelial cell cultures were prepared as described in Example 1. Cisplatin was added to each test well (1 μg/ml final concentration of cisplatin); two hours later 5 μl of an EPO preparation ranging from 0.15 to 20 U/ml final concentration was added. Endothelial cell viability was assessed using the MTS/PMS colorimetric assay described in Example 1. Results were compared to control wells (endothelial cells treated with 1μg/ml cisplatin alone).

Results are provided in **Figure 3**, and show that a biphasic response was observed when EPO was added to cell cultures after the addition of cisplatin. Endothelial cell cultures treated with from 0.15 to 5 U/ml of EPO were protected from the damaging effects of cisplatin when EPO was added two hours following cisplatin exposure. The number of viable cells after treatment with 1.25 U/ml EPO after cisplatin exposure was 34% greater than that of controls. In contrast, cell viability in the presence of 10 to 20 U/ml EPO administered two hours after cisplatin exposure was reduced over that seen in controls (cisplatin only).

EXAMPLE 5

Effects of EPO on Endothelial Cells Administered Prior to Cisplatin Exposure

In this experiment, serial dilutions of EPO were added to endothelial cell cultures two hours before the cultures were exposed to cisplatin.

Endothelial cell cultures were prepared as described in Example 1. Each test well received 5 μl of

an EPO preparation ranging from 0.15 to 20 U/ml EPO; two hours later cisplatin was added to each test well (5μl of 1 μg/ml cisplatin). Endothelial cell viability was assessed using the MTS/PMS colorimetric assay described in Example 1. Results were compared to control wells (endothelial cells treated with 1 μg/ml cisplatin alone).

Results are provided in **Figure 4**, and show a reduction in the number of viable endothelial cells after exposure to EPO two hours prior to cisplatin exposure (compared to control cells exposed only to cisplatin). Cell proliferation and viability was decreased by as much as 81% compared to controls. The inhibition was dose dependent; EPO concentrations as low as 5 and 2.5 U/ml reduced cell growth by 58% and 48%, respectively, compared to controls.

The foregoing is illustrative of the present invention and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

THAT WHICH IS CLAIMED IS:

1. A method of reducing endothelial injury in a subject caused by administration of a chemotherapeutic agent to the subject, comprising administering an endothelial-protecting amount of erythropoietin in conjunction with the administration of the chemotherapeutic agent.

2. A method according to claim 1 wherein said endothelial-protecting amount of erythropoietin is administered simultaneously with said chemotherapeutic agent.

3. A method according to claim 1 wherein said endothelial-protecting amount of erythropoietin is administered prior to said chemotherapeutic agent.

4. A method according to claim 1 wherein said endothelial-protecting amount of erythropoietin is administered after said chemotherapeutic agent.

5. The method of claim 1 where said chemotherapeutic agent is cisplatin.

6. A method of enhancing endothelial cell inhibition in a subject treated with a chemotherapeutic agent, comprising administering to the subject an endothelial-inhibiting amount of erythropoietin in conjunction with the administration of the chemotherapeutic agent.

7. A method according to claim 6 wherein said endothelial-inhibiting amount of erythropoietin is administered simultaneously with said chemotherapeutic agent.

8. A method according to claim 6 wherein said endothelial-inhibiting amount of erythropoietin is administered prior to said chemotherapeutic agent.

9. A method according to claim 6 wherein said endothelial-inhibiting amount of erythropoietin is administered after said chemotherapeutic agent.

10. A method according to claim 6 wherein said chemotherapeutic agent is cisplatin.

11. A method according to claim 6 wherein said subject has a neoplastic growth selected from the group consisting of cerebellar hemangioblastoma, ductal carcinoma of the breast, and squamous cell cancer of the larynx.

12. A method of treating a solid vascularized tumor in a subject in need of such treatment, comprising administering an antineoplastic chemotherapeutic agent in conjunction with an endothelial-inhibiting amount of erythropoietin.

13. A method according to claim 12, wherein said endothelial-inhibiting amount of erythropoietin is administered simultaneously with said chemotherapeutic agent.

14. A method according to claim 12 wherein said endothelial-inhibiting amount of erythropoietin is administered prior to said chemotherapeutic agent.

15. A method according to claim 12 wherein said endothelial-inhibiting amount of erythropoietin is administered after said chemotherapeutic agent.

16. A method of treating endothelial injury caused by mechanical damage, exposure to radiation, inflammation, heart disease or cancer, in a subject in need of such treatment, comprising administering an endothelial-protecting amount of erythropoietin to said subject.

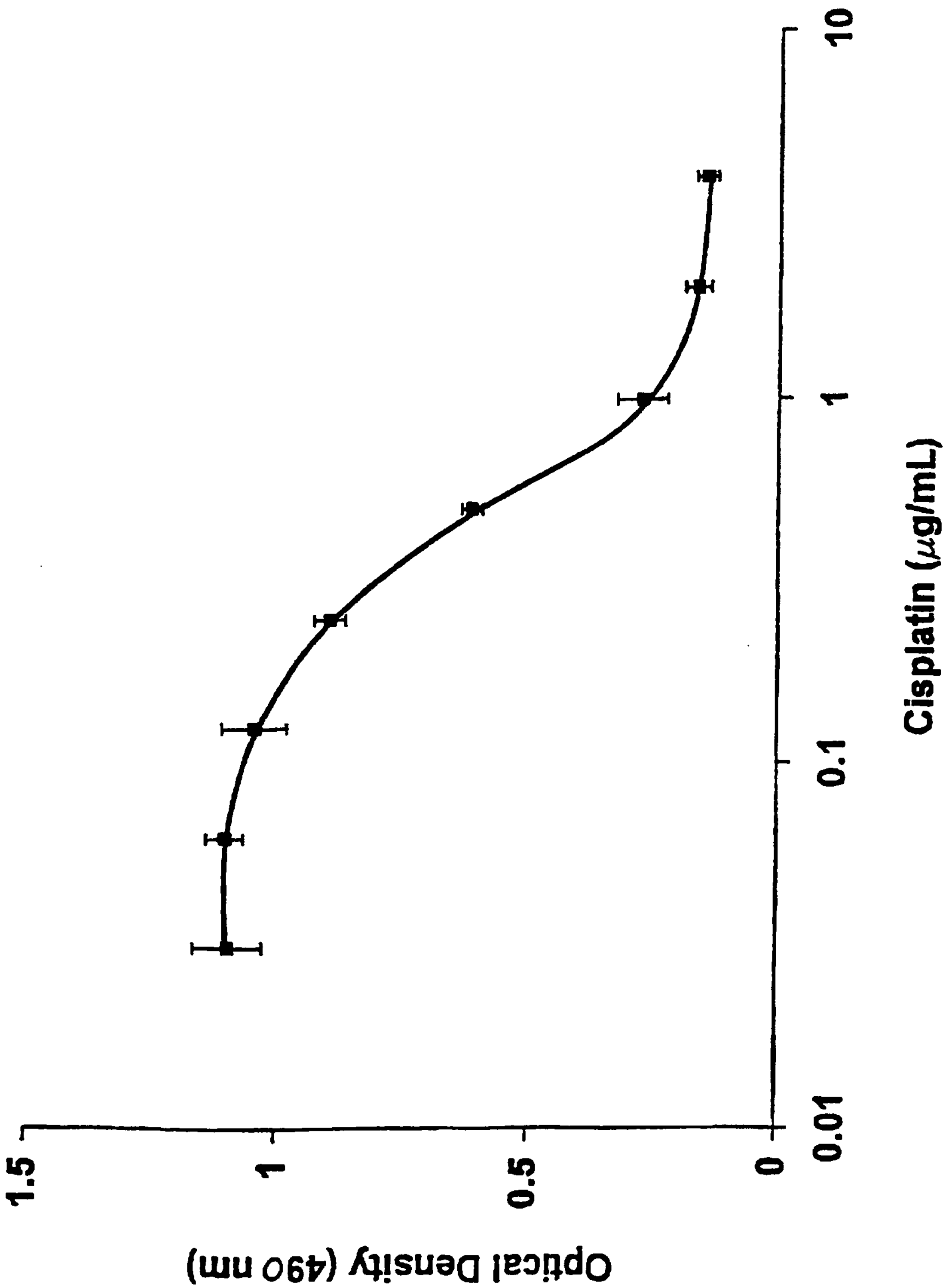
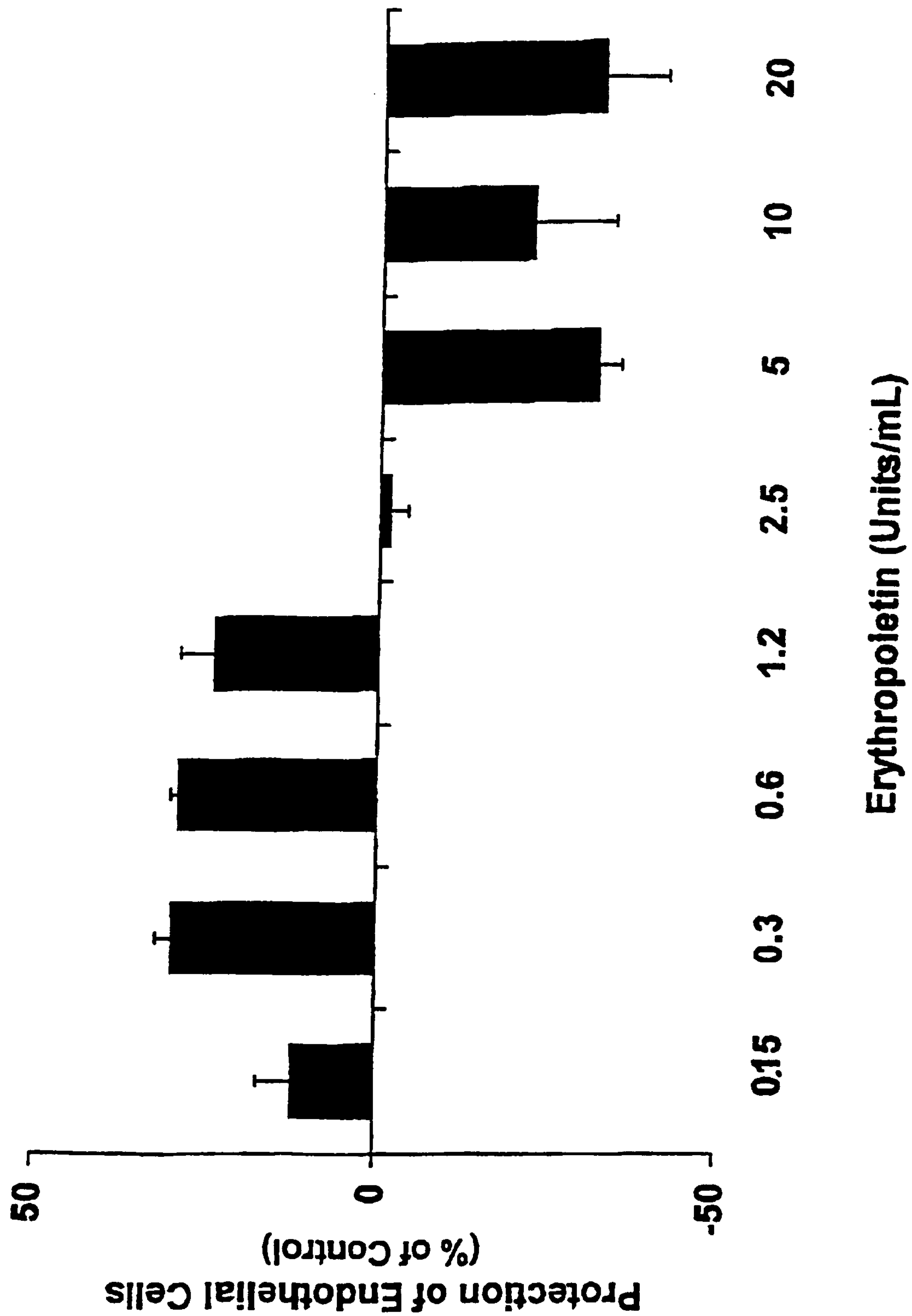


Figure 1

Figure 2.


Protection of Endothelial Cells
(% of Control)
50
0
-50
0.15
0.3
0.6
1.2
2.5
5
10
20
Erythropoietin (Units/mL)

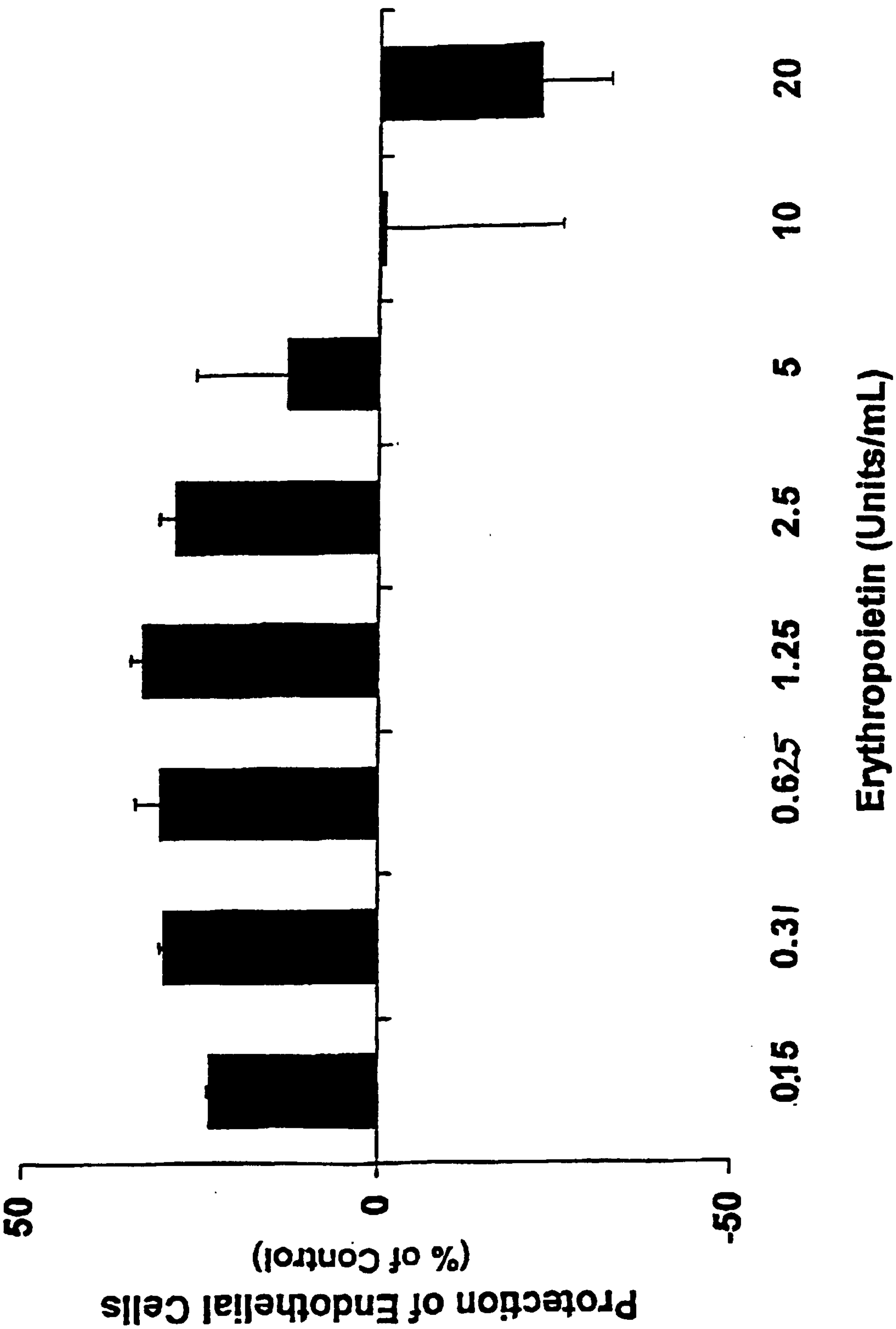
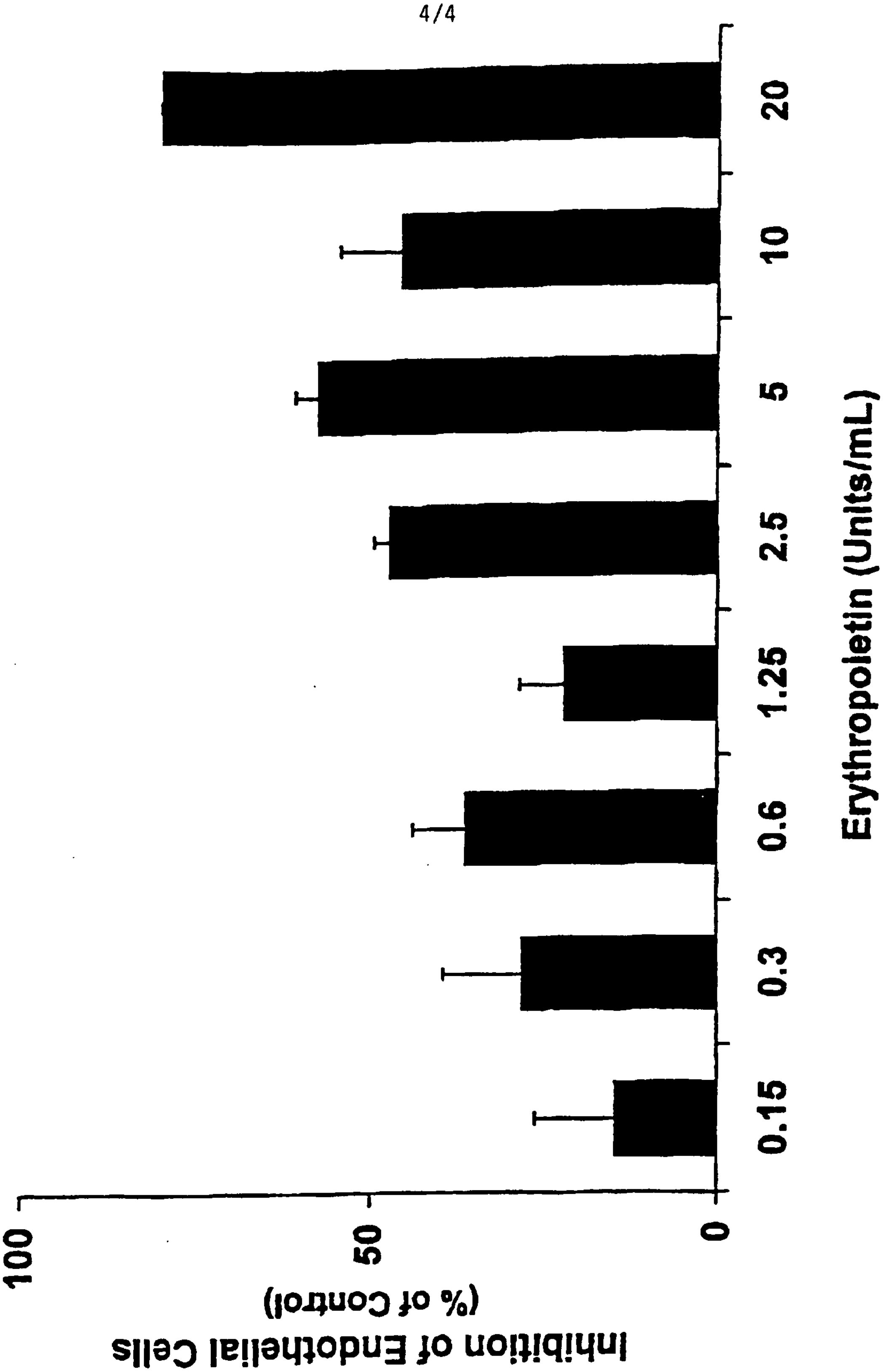

50
0
-50
Protection of Endothelial Cells
(% of Control)
0.15
0.31
0.625
1.25
2.5
5
10
20
Erythropoietin (Units/mL)

Figure 3

Figure 4


Inhibition of Endothelial Cells
(% of Control)
100
50
0
0.15
0.3
0.6
1.25
2.5
5
10
20
Erythropoietin (Units/mL)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/15966

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 37/18; A61K 38/00
US CL : 514/2, 12
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CAZZOLA, M. Erythropoietin: biology and clinical applications. Trends in Experimental and Clinical Medicine, 1993, Vol. 3, pages 344-361, see entire article.	1-16
A	KURIYAMA et al. Evidence for Amelioration of Endothelial Cell Dysfunction by Erythropoietin Therapy in Predialysis Patients. Am. J. Hyperten. 1996, Vol. 9, No. 5, pages 426-431, see the entire article.	1-16

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 10 OCTOBER 1997	Date of mailing of the international search report 24 OCT 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SUSAN UNGAR Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, CAPLUS, CA, CANCERLIT, LIFESCI, BIOTECHDS, BIOSIS, BIOBUSINESS
search terms: erythropoietin, endothelial cells chemotherapy, treatment, radiation, heart disease, cancer, tumor, inhibition

JP006867

WO9921966.pdf
06/27/08 09:42 PM

WO9921966

Publication Title:

ERYTHROPOIETIN-MEDIATED NEUROGENESIS

Abstract:

Abstract of WO9921966

Methods are described for the production of neurons or neuronal progenitor cells. Multipotent neural stem cells are proliferated in the presence of growth factors and erythropoietin which induces the generation of neuronal progenitor cells. The erythropoietin may be exogenously applied to the multipotent neural stem cells, or alternatively, the cells can be subjected to hypoxic insult which induces the cells to express e34 erythropoietin. Data supplied from the esp@cenet database - Worldwide

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 5/06, 5/08, 5/00, C07K 14/505	A1	(11) International Publication Number: WO 99/21966 (43) International Publication Date: 6 May 1999 (06.05.99)

(21) International Application Number: PCT/CA98/00991

(22) International Filing Date: 23 October 1998 (23.10.98)

(30) Priority Data:
60/063,040 24 October 1997 (24.10.97) US

(71) Applicant: NEUROSPHERES HOLDINGS LTD. [CA/CA]; Suite 204, 609 14th Street N.W., Calgary, Alberta T2N 2A1 (CA).

(72) Inventors: WEISS, Samuel; 4540 Chapel Road N.W., Calgary, Alberta T2L 1A6 (CA). SOROKAN, S., Todd; 433 Marlborough Way N.E., Calgary, Alberta T2A 5H5 (CA).

(74) Agent: CALDWELL, Roseann; Bennett Jones, 4500 Bankers Hall East, 855 2nd Street S.W., Calgary, Alberta T2P 4K7 (CA).

(81) Designated States: AU, CA, JP, NO, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published

With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ERYTHROPOIETIN–MEDIATED NEUROGENESIS

(57) Abstract

Methods are described for the production of neurons or neuronal progenitor cells. Multipotent neural stem cells are proliferated in the presence of growth factors and erythropoietin which induces the generation of neuronal progenitor cells. The erythropoietin may be exogenously applied to the multipotent neural stem cells, or alternatively, the cells can be subjected to hypoxic insult which induces the cells to express erythropoietin.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece		Republic of Macedonia	**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's	**NZ**	New Zealand		
CM	Cameroon		Republic of Korea	**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

ERYTHROPOIETIN-MEDIATED NEUROGENESIS

FIELD OF THE INVENTION

This invention relates to methods of influencing multipotent neural stem cells to produce progeny that differentiate into neurons by exposing the stem cells and their progeny to erythropoietin.

BACKGROUND OF THE INVENTION

Neurogenesis in mammals is complete early in the postnatal period. Cells of the adult mammalian CNS have little or no ability to undergo mitosis and generate new neurons. While a few mammalian species (e.g. rats) exhibit the limited ability to generate new neurons in restricted adult brain regions such as the dentate gyrus and olfactory bulb (Kaplan, J. Comp. Neurol., 195:323, 1981; Bayer, N.Y. Acad. Sci., 457:163, 1985), the generation of new CNS neurons in adult primates does not normally occur (Rakic, Science, 227:1054, 1985). This inability to produce new nerve cells in most mammals (and especially primates) may be advantageous for long-term memory retention; however, it is a distinct disadvantage when the need to replace lost neuronal cells arises due to injury or disease.

The role of stem cells in the adult is to replace cells that are lost by natural cell death, injury or disease. Until recently, the low turnover of cells in the mammalian CNS together with the inability of the adult mammalian CNS to generate new neuronal cells in response to the loss of cells following injury or disease had led to the assumption that the adult mammalian CNS does not contain multipotent neural stem cells. The critical identifying feature of a stem cell is its ability to exhibit self-

renewal or to generate more of itself. The simplest definition of a stem cell would be a cell with the capacity for self-maintenance. A more stringent (but still simplistic) definition of a stem cell is provided by Potten and Loeffler (Development, 110:1001, 1990) who have defined stem cells as "undifferentiated cells capable of a) proliferation, b) self-maintenance, c) the production of a large number of differentiated functional progeny, d) regenerating the tissue after injury, and e) a flexibility in the use of these options."

CNS disorders encompass numerous afflictions such as neurodegenerative diseases (e.g. Alzheimer's and Parkinson's), acute brain injury (e.g. stroke, head injury, cerebral palsy) and a large number of CNS dysfunctions (e.g. depression, epilepsy, and schizophrenia). Degeneration in a brain region known as the basal ganglia can lead to diseases with various cognitive and motor symptoms, depending on the exact location. The basal ganglia consists of many separate regions, including the striatum (which consists of the caudate and putamen), the globus pallidus, the substantia nigra, substantia innominate, ventral pallidum, nucleus basalis of Meynert, ventral tegmental area and the subthalamic nucleus. Many motor deficits are a result of neuronal degeneration in the basal ganglia. Huntington's Chorea is associated with the degeneration of neurons in the striatum, which leads to involuntary jerking movements in the host. Degeneration of a small region called the subthalamic nucleus is associated with violent flinging movements of the extremities in a condition called ballismus, while degeneration in the putamen and globus pallidus is associated with a condition of slow writhing movements or athetosis. Other forms of neurological impairment can occur as a result of neural degeneration, such as cerebral palsy, or as a result of CNS trauma, such as stroke and epilepsy.

In recent years neurodegenerative disease has become an important concern due to the expanding elderly population which is at greatest risk for these disorders. These diseases, which include Alzheimer's Disease and Parkinson's Disease, have been linked to the degeneration of neuronal cells in particular locations of the CNS, leading to the inability of these cells or the brain region to carry out their intended

function. In the case of Alzheimer's Disease, there is a profound cellular degeneration of the forebrain and cerebral cortex. In addition, upon closer inspection, a localized degeneration in an area of the basal ganglia, the nucleus basalis of Meynert, appears to be selectively degenerated. This nucleus normally sends cholinergic projections to the cerebral cortex which are thought to participate in cognitive functions including memory. In the case of Parkinson's Disease, degeneration is seen in another area of the basal ganglia, the substantia nigra par compacta. This area normally sends dopaminergic connections to the dorsal striatum which are important in regulating movement. Therapy for Parkinson's Disease has centered upon restoring dopaminergic activity to this circuit through the use of drugs.

In addition to neurodegenerative diseases, acute brain injuries often result in the loss of neurons, the inappropriate functioning of the affected brain region, and subsequent behavior abnormalities.

To date, treatment for CNS disorders has been primarily via the administration of pharmaceutical compounds. Unfortunately, this type of treatment has been fraught with many complications including the limited ability to transport drugs across the blood-brain barrier and the drug-tolerance which is acquired by patients to whom these drugs are administered long-term. For instance, partial restoration of dopaminergic activity in Parkinson's patients has been achieved with levodopa, which is a dopamine precursor able to cross the blood-brain barrier. However, patients become tolerant to the effects of levodopa, and therefore, steadily increasing dosages are needed to maintain its effects. In addition, there are a number of side effects associated with levodopa such as increased and uncontrollable movement.

Recently, the concept of neurological tissue grafting has been applied to the treatment of neurological diseases such as Parkinson's Disease. Neural grafts may avert the need not only for constant drug administration, but also for complicated drug delivery systems which arise due to the blood-brain barrier. However, there are limitations to this technique as well. First, cells used for transplantation which

carry cell surface molecules of a differentiated cell from another host can induce an immune reaction in the host. In addition, the cells must be at a stage of development where they are able to form normal neural connections with neighboring cells. For these reasons, initial studies on neurotransplantation centered on the use of fetal cells. Several studies have shown improvements in patients with Parkinson's Disease after receiving implants of fetal CNS tissue. Implants of embryonic mesencephalic tissue containing dopamine cells into the caudate and putamen of human patients was shown by Freed *et al.* (N Engl J Med 327:1549-1555 (1992)) to offer long-term clinical benefit to some patients with advanced Parkinson's Disease. Similar success was shown by Spencer *et al.* (N Engl J Med 327:1541-1548 (1992)). Widner *et al.* (N Engl J Med 327:1556-1563 (1992)) have shown long-term functional improvements in patients with MPTP-induced Parkinsonism that received bilateral implantation of fetal mesencephalic tissue. Perlow, *et al.* describe the transplantation of fetal dopaminergic neurons into adult rats with chemically induced nigrostriatal lesions in "Brain grafts reduce motor abnormalities produced by destruction of nigrostriatal dopamine system," Science 204:643-647 (1979). These grafts showed good survival, axonal outgrowth and significantly reduced the motor abnormalities in the host animals.

While the studies noted above are encouraging, the use of large quantities of aborted fetal tissue for the treatment of disease raises ethical considerations and political obstacles. There are other considerations as well. Fetal CNS tissue is composed of more than one cell type, and thus is not a well-defined source of tissue. In addition, there are serious doubts as to whether an adequate and constant supply of fetal tissue would be available for transplantation. For example, in the treatment of MPTP-induced Parkinsonism (Widner *supra*) tissue from 6 to 8 fresh fetuses were required for implantation into the brain of a single patient. There is also the added problem of the potential for contamination during fetal tissue preparation. Moreover, the tissue may already be infected with a bacteria or virus, thus requiring expensive diagnostic testing for each fetus used. However, even diagnostic testing might not uncover all infected tissue. For example, the diagnosis of HIV-free tissue is not guaranteed because antibodies to the virus are generally not present until several

weeks after infection.

While currently available transplantation approaches represent a significant improvement over other available treatments for neurological disorders, they suffer from significant drawbacks. The inability in the prior art of the transplant to fully integrate into the host tissue, and the lack of availability of neuronal cells in unlimited amounts from a reliable source for grafting are, perhaps, the greatest limitations of neurotransplantation. A well-defined, reproducible source of neural cells has recently been made available. It has been discovered that multipotent neural stem cells, capable of producing progeny that differentiate into neurons and glia, exist in adult mammalian neural tissue. (Reynolds and Weiss, Science 255:1707 (1992)). Methods have been provided for the proliferation of these stem cells to provide large numbers of neural cells that can differentiate into neurons and glia (See. U.S. Pat. No. 5,750,376, and International Application No. WO 93/01275). Various factors can be added to neural cell cultures to influence the make-up of the differentiated progeny of multipotent neural stem cell progeny, as disclosed in published PCT application WO 94/10292. Additional methods for directing the differentiation of the stem cell progeny would be desirable.

SUMMARY OF THE INVENTION

A method of producing neurons or neuronal progenitor cells which can be used for transplantation or other purposes is described. The method comprises inducing multipotent neural stem cells to produce neuronal progenitor cells by proliferating the multipotent neural stem cells in the presence of growth factors and erythropoietin. The erythropoietin may originate from the population of neural cells by subjecting the cells to hypoxic insult which induces neural cells to express erythropoietin. Alternatively, the erythropoietin may be provided exogenously.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the term multipotent or oligopotent neural stem cell refers to an undifferentiated cell which is capable of self-maintenance. Thus, in essence, a stem cell is capable of dividing without limit. The non-stem cell progeny of a multipotent

neural stem cell are termed "progenitor cells." A distinguishing feature of a progenitor cell is that, unlike a stem cell, it has limited proliferative ability and thus does not exhibit self-maintenance. It is committed to a particular path of differentiation and will, under appropriate conditions, eventually differentiate. A neuronal progenitor cell is capable of a limited number of cell divisions before giving rise to differentiated neurons. A glial progenitor cell likewise is capable of a limited number of cell divisions before giving rise to astrocytes or oligodendrocytes. A neural stem cell is multipotent because its progeny include both neuronal and glial progenitor cells and thus is capable of giving rise to neurons, astrocytes, and oligodendrocytes.

Various factors can be added to neural cell cultures to influence the make-up of the differentiated progeny of multipotent neural stem cell progeny, as disclosed in WO 94/10292. It has now been found that erythropoietin (EPO), a hormone thought to influence the differentiative pathway of hematopoietic stem cells and/or their progeny, can increase the number of neuronal progeny that are generated from proliferated multipotent neural stem cells. Multipotent neural stem cells proliferated in the presence of EPO produce a greater percentage of neuronal progenitor cells than multipotent neural stem cells proliferated in the absence of EPO.

Multipotent neural stem cells can be obtained from embryonic, juvenile, or adult mammalian neural tissue (e.g. mouse and other rodents, and humans and other primates) and can be induced to proliferate *in vitro* or *in vivo* using the methods disclosed in published PCT application WO 93/01275 and U.S. Pat. No. 5,750,376. Briefly, the administration of one or more growth factors can be used to induce the proliferation of multipotent neural stem cells. Preferred proliferation-inducing growth factors include epidermal growth factor (EGF), amphiregulin, acidic fibroblast growth factor (aFGF or FGF-1), basic fibroblast growth factor (bFGF or FGF-2), transforming growth factor alpha (TGFα), and combinations thereof. For the proliferation of multipotent neural stem cells *in vitro*, neural tissue is dissociated and the primary cell cultures are cultured in a suitable culture medium, such as the serum-free defined medium described in Example 1. A suitable proliferation-

inducing growth factor, such as EGF (20 ng/ml) is added to the culture medium to induce multipotent neural stem cell proliferation.

In the absence of substrates that promote cell adhesion (e.g. ionically charged surfaces such as poly-L-lysine and poly-L-ornithine coated and the like), multipotent neural stem cell proliferation can be detected by the formation of clusters of undifferentiated neural cells termed "neurospheres", which after several days in culture, lift off the floor of the culture dish and float in suspension. Each neurosphere results from the proliferation of a single multipotent neural stem cell and is comprised of daughter multipotent neural stem cells and neural progenitor cells. The neurospheres can be dissociated to form a suspension of undifferentiated neural cells and transferred to fresh growth-factor containing medium. This re-initiates proliferation of the stem cells and the formation of new neurospheres. In this manner, an unlimited number of undifferentiated neural stem cell progeny can be produced by the continuous culturing and passaging of the cells in suitable culture conditions.

Various procedures are disclosed in WO 94/10292 and U.S. Pat. No. 5,750,376 which can be used to induce the proliferated neural stem cell progeny to differentiate into neurons, astrocytes and oligodendrocytes. To increase the number of neuronal progenitor cells that are produced by the multipotent neural stem cells, the proliferating stem cells can be exposed to EPO. The EPO can be exogenously added at concentrations from about 0.1 to 10 units/ml. Alternatively, the neural cells can be induced to express endogenous EPO by subjecting the cells to hypoxic insult. Subsequent differentiation of the progenitor cell progeny results in at least a two-fold increase in the numbers of neurons generated compared to progeny of stem cells that have not been exposed to EPO, as evidenced by immunocytochemical analysis. Differentiation of cells that have not been exposed to endogenously added EPO or hypoxic insult typically results in a population of cells containing about 3% neurons. The percentage of neurons increases to about 6% with hypoxia treatment, and to about 10% with exposure to exogenous EPO, with the percentage of astrocytes and oligodendrocytes remaining about the same as the control

populations.

Washout experiments, in which the growth factor/EPO medium is removed after 24 hours and changed to regular growth factor-containing medium, reveals that the EPO instructs the stem cells prior to their first cell division, to produce more neurons. The continued presence of EPO after the initial 24 hours does not result in a further increase in the numbers of neurons over cultures subjected to EPO for a 24 hour period.

The ability to manipulate the fate of the differentiative pathway of the multipotent neural stem cell progeny to produce more neuronal progenitor cells and neurons is beneficial. Cell cultures that contain a higher percentage of neuronal progenitor cells and/or neurons will be useful for screening the effects of various drugs and other agents on neuronal cells. Methods for screening the effect of drugs on cell cultures are well known in the art and are also disclosed in U.S. Pat. No. 5,750,376.

Cell cultures with an enriched neuronal-progenitor cell and/or neuron population can be used for transplantation to treat various neurological injuries, diseases or disorders. The neuronal progenitor cells or neurons or a combination thereof can be harvested and transplanted into a patient needing neuronal augmentation. Neuronal progenitor cells are particularly suitable for transplantation because they are still undifferentiated and, unlike differentiated neurons, there are no branched processes which can be damaged during transplantation procedures. Once transplanted, the neuronal progenitor cells differentiate *in situ* into new, functioning neurons. Suitable transplantation methods are known in the art and are disclosed in U.S. Pat. No. 5,750,376.

Alternatively, a patient's endogenous multipotent neural stem cells could be induced to proliferate *in situ* to produce neuronal progenitor cells by administering to the patient a composition comprising one or more growth factors which induces the patient's neural stem cells to proliferate and EPO which instructs the proliferating

neural stem cells to produce neuronal progenitor cells which eventually differentiate into neurons. Suitable methods for administering a composition to a patient which induces the *in situ* proliferation of the patient's stem cells are disclosed in U.S. Pat. No. 5,750,376.

All cited references, patents and applications are herein incorporated in their entireties by reference.

EXAMPLE 1: Multipotent neural stem cell proliferation

Striata from 14-day-old mouse embryos were removed using sterile procedure. Tissue was mechanically dissociated into serum-free medium composed of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F-12 nutrient (Gibco). Dissociated cells were centrifuged, the supernatant aspirated, and the cells resuspended at a concentration of about 1 x 10^5 cell/ml in a serum-free medium, referred to herein as "complete medium" composed of DMEM/F-12 (1:1) including glucose (0.6%), glutamine (2 μM), sodium bicarbonate (3 mM), and HEPES (4-[2hydroxyethyl]-1-piperazineethanesulfonic acid) buffer (5 mM) (all from Sigma except glutamine [Gibco]). A defined hormone mix and salt mixture (Sigma) that included insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (60 μM), and selenium chloride (30 nM) was used in place of serum. The complete medium was supplemented with 20 ng/ml of EGF (Collaborative Research). Cells were seeded in a T25 culture flask and housed in an incubator at 37°C, 100% humidity, 95% air/5% CO_2. Stem cells within the cultures began to proliferate within 3-4 days and due to a lack of substrate lifted off the floor of the flask and continued to proliferate in suspension forming neurospheres.

EXAMPLE 2: Hypoxia-induced neurogenesis

After 6 days *in vitro* primary neurospheres formed using the methods described in Example 1 were dissociated and were replated in EGF-containing medium. After 24 hours, the cells were exposed to a modest hypoxic insult by decreasing the concentration of oxygen in the culture medium for varying lengths of time (from 1 to 12 hours) from normal levels of 135 mmHg to 30-40 mmHg. The cells were then

cultured in the EGF-containing complete medium described in Example 1 in 95% air/5% CO_2 for 7 days. Hypoxia did not prevent multipotent neural stem cell proliferation, as evidenced by the formation of secondary neurospheres. The number of progeny produced from hypoxia-treated stem cells was the same as that in control cultures not subjected to hypoxic insult.

Secondary neurospheres generated from untreated or hypoxia-treated stem cells were dissociated into single cells and induced to differentiate by plating between 0.5 x 10^6 and 1.0 x 10^6 cells onto poly-L-ornithine-coated (15 μg/ml) glass coverslips in 24 well Nuclon (1.0 ml/well) culture dishes in EGF-free complete medium optionally supplemented with 1% FBS. After 7 days, the cells were assayed using immunocytochemical analysis for the presence of neurons. Cultures that had been subjected to hypoxic conditions for 1 to 4 hours had approximately a two-fold increase in the percentage of neurons (approx. 6%) over control cultures (approx. 3%). Cultures subjected to 4 to 8 hours of hypoxia had fewer neurons produced and cultures subjected to about 12 hours of hypoxia had normal levels (approx. 3%). The hypoxic insult induced a rapid up-regulation of hypoxia-induced factor (HIF) in the multipotent neural stem cell progeny. HIP is a transcription factor for EPO. The 4-hour hypoxia-induced increase in neurogenesis could be blocked by the addition of an EPO-neutralizing antibody at 3 μg/ml.

EXAMPLE 3: Erythropoietin-induced neurogenesis

After 6 days *in vitro* primary neurospheres formed using the methods described in Example 1 were dissociated and replated in complete medium containing EGF at 20 ng/ml and human recombinant EPO at 0.1 to 10 units/ml for either 24 hours or 7 days under normal oxygen conditions (95% air/5% CO_2; 135 mmHg). In both cases, immunocytochemistry revealed an EPO dose-dependent three-fold increase in the numbers of neurons generated.

WHAT IS CLAIMED IS:

1. A method of producing neurons from a population of neural cells containing at least one multipotent neural stem cell comprising inducing said at least one multipotent neural stem cell to proliferate in the presence of erythropoietin to produce neuronal progenitor cells and allowing said neuronal progenitor cells to differentiate into neurons.

2. The method of claim 1 wherein said population of neural cells is induced to express said erythropoietin by subjecting said population of neural cells to hypoxic insult.

3. The method of claim 1 wherein said erythropoietin is exogenously added.

4. The method of claim 1 wherein at least one exogenously added growth factor induces said at least one multipotent neural stem cell to proliferate.

5. The method of claim 4 wherein said exogenously added growth factor is epidermal growth factor.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 98/00991

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/06 C12N5/08 C12N5/00 C07K14/505

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	T. SOROKAN AND S. WEISS: "Effects of hypoxia on neuronal production from embryonic murine CNS stem cells" MOLECULAR BIOLOGY OF THE CELL, vol. 7, no. suppl, 1996, page p371A XP002098356 see abstract 1843 ---	1,2,4
A	S. MASUDA ET AL., : "Insulin-like growth factors and insulin stimulate erythropoietin production in primary cultured astrocytes" BRAIN RESEARCH, vol. 746, no. 1-2, 23 January 1997, pages 63-70, XP002098357 see the whole document ---	1,2,4

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 April 1999	16/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mateo Rosell, A.M.

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	S. E. JUUL AND R.D. CHRISTENSEN: "Erythropoietin (EPO) may promote neuronal survival following hypoxia, by repressing apoptosis" PEDIATRIC RESEARCH, vol. 41, no. 4 part 2, 2 May 1997, page p291A XP002098358 see abstract 1734 ---	1,2
A	S. MASUDA ET AL., : "A novel site of erythropoietin production: Oxygen-dependant production in cultured rat astrocytes" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 30, 1994, pages 19488-19493, XP002098359 see the whole document ---	1,4
A	H.H. MARTI ET AL., : "Detection of erythropoietin in human liquor: intrinsic erythropoietin production in the brain" KIDNEY INTERNATIONAL, vol. 51, no. 2, February 1997, pages 416-418, XP002098360 see the whole document ---	1,2
A	M. DIGICAYLIOGLU ET AL., : "Localization of specific erythropoietin binding sites in defined areas of the mouse brain." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 92, no. 9, 1995, pages 3717-3720, XP002098361 see the whole document ---	1,2,4
A	S.WEISS ET AL., : "Is there a neural stem cell in the mammalian forebrain?" TRENDS IN NEUROSCIENCES, vol. 19, no. 9, 1996, pages 387-393, XP002098734 see the whole document ---	1,21
A	WO 95 03821 A (O'BRIEN JOHN) 9 February 1995 see page 10, line 1 - page 11, line 1 see page 12, line 35 - page 13, line 16; table 2 see page 14, line 13-18 ---	1,3,4

-/--

1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 15226 A (NEUROSPHERES HOLDINGS LTD ;WEISS SAMUEL (CA); REYNOLDS BRENT A (CA) 23 May 1996 see abstract see page 8, line 25 - page 9, line 16 see page 15, line 3 - page 17, line 21; examples 1-7	1,4,5
A	WO 95 13364 A (CRAIG CONSTANCE ;KOOY DEREK V D (CA); MORSHEAD CINDI (CA); WEISS S) 18 May 1995 see abstract see page 3, line 1 - page 4, line 2 see page 12, line 5-27 see page 18, line 5 - page 20, line 16	1,4,5
A	WO 94 10292 A (NEUROSPHERES LTD) 11 May 1994 cited in the application see abstract see page 14, line 8-28; example 1 see page 11, line 7 - page 12, line 10	1,4,5
P,A	C. BAUER : "Upregulation of erythropoietin by hypoxia in the brain of mammals" WIENER KLINISCHE WOCHENSCHRIFT, vol. 109, no. 12-13, 1997, page p535 XP002098362 see abstract	1,2
P,X	S.E. JUUL ET AL., : "Erythropoietin and erythropoietin receptor in the developing human central nervous system" PEDIATRIC RESEARCH, vol. 43, no. 1, 1 January 1998, pages 40-49, XP002098363 see specially abstract and discussion.	1,2
P,X	T. SOROKAN AND S. WEISS : "Erythropoietin mediates increased neurogenesis by embryonic CNS stem cells following a modest hypoxic insult" SOCIETY FOR NEUROSCIENCES ABSTRACTS, vol. 23, no. 1-2, 1997, page p320 XP002098364 see abstract 131.12	1,2,4

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/CA 98/00991

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9503821 A	09-02-1995	US 5571787 A	05-11-1996
		US 5700909 A	23-12-1997
		AU 7515494 A	28-02-1995
		CA 2168029 A	09-02-1995
		EP 0720482 A	10-07-1996
		JP 9503999 T	22-04-1997
		US 5696080 A	09-12-1997
		US 5714459 A	03-02-1998
WO 9615226 A	23-05-1996	AU 3836795 A	06-06-1996
		CN 1170435 A	14-01-1998
		EP 0792350 A	03-09-1997
		FI 971956 A	04-07-1997
		JP 10509592 T	22-09-1998
		NO 972171 A	07-07-1997
		US 5750376 A	12-05-1998
		US 5851832 A	22-12-1998
WO 9513364 A	18-05-1995	AU 697894 B	22-10-1998
		AU 8056194 A	29-05-1995
		CA 2175992 A	18-05-1995
		CN 1141058 A	22-01-1997
		EP 0728194 A	28-08-1996
		FI 961855 A	04-06-1996
		JP 9507747 T	12-08-1997
		NO 961859 A	03-07-1996
		US 5750376 A	12-05-1998
		US 5851832 A	22-12-1998
WO 9410292 A	11-05-1994	AU 4924197 A	12-03-1998
		AU 5367694 A	24-05-1994
		EP 0669973 A	06-09-1995
		FI 952022 A	27-04-1995
		JP 8502652 T	26-03-1996
		NO 951617 A	27-04-1995
		WO 9416718 A	04-08-1994
		US 5750376 A	12-05-1998
		US 5851832 A	22-12-1998